CHARACTERIZATION OF A TRANSGENIC MOUSE EXPRESSING THE HSV-1 LATENCY ASSOCIATED TRANSCRIPT

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2004

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ANNE M. GUSSOW

To my husband, Karl, who is my best cheerleader,
and my son, Seth, who is too young to understand.

ACKNOWLEDGMENTS

I would first like to thank my mentor, Dr. David Bloom, for his guidance and direction in the completion of this work and for providing me with the opportunity to complete my doctoral studies at the University of Florida. I would also like to thank my committee, Dr. Richard Condit, Dr. Sue Moyer, and Dr. Paul Reier, for their useful discussions and suggestions to this project.

I would like to thank Dr. Robert Bonneau for encouraging me to apply to graduate school and for giving me a start in my research career, and Dr. Eddie Castaneda for helping to ease my transition from Arizona State to the University of Florida.

I would like to express my gratitude to the people who have worked with me in Dave's lab over the years, both the ASU group, Rick Jarman, Robert Tran, Jerry O'Neil, Niki Kubat, Melanie Paquette, and Lee Gary, as well as the UF group, Tony Amelio, Zane Zeier, Nicole Giordiani, Peterjon McAnany and Loretta Arrue. Each of them has brought a special quality to the lab that will not be forgotten.

The biggest acknowledgment goes to my husband, Karl, and my son, Seth. Without the love and support of both of them this goal would have fallen short a long time ago. I thank Karl for the 2500 miles of I-10, I hope the journey has been worth the sacrifices that he has made as a result. Although Seth is too young to understand any of this, coming home to his simple outlook on life has put things into perspective on many occasions.

I also want to thank my mother who has listened to the ups and downs all along the way and always been supportive of me no matter what. It's been a long journey around the country and I'm finally going to "settle down" in one place. To my brothers, Marty and Karl, who haven't understood all of what my research is about, thanks for listening and pretending to understand. To Cathy Kostick, who has been like a sister to me, thank you for always being there through the years, in good times and in bad, your friendship means more to me than I can express.

Last but certainly not least, I would like to thank my friends who have been there for me every time that I was ready to give up; those of you who reminded me to do the next right thing, take it one day at a time and put first things first. In no particular order: Corliss, Aileen, Julie, Betty, Sheila, Eve, Heather, Leona, Martha Ann, Jennifer, Sarah, Claire, Alice, Polly, Joan, Mary Ellen, Marty, Ted, Steve, Dale, Rosemary, Julia, Casey, Diedre, Buster, BJ, Warren, Jim, Walt, Dan, and Joe; you are each a special part of my life and of making this dream come true. And to whomever I have forgotten, you know who you are, and I thank you.

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KEY TO SYMBOLS

°C	degrees Celsius
AL	antisense to LAT
ANOVA	analysis of variance
β-gal	beta galactosidase
bp	base pair
Br	brain
$CaCl_2$	calcium chloride
cDNA	copy deoxynucleic acid
cm	centimeter
CMV	cytomegalovirus
CO_2	carbon dioxide
CPE	cytopathic effect
cpm	counts per minute
CRE	cyclic AMP response element
CTL	cytotoxic T lymphocyte
dATP	adenosine triphosphate nucleotide
dCTP	cytosine triphosphate nucleotide
dGTP	guanine triphosphate nucleotide
dTTP	thymine triphosphate nucletide
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
EDTA	ethylenediaminetetraacetic acid
TE	TRIS/ EDTA buffer
F	foot
FFLB	formaldehyde loading buffer
FHP	formamide prehybridization/ hybridization buffer
fmol	femtomole
g	centrifugal force
HCl	hydrochloric acid
HSV	Herpes Simplex Virus
HSV-1	Herpes Simplex Virus Type 1
HSV-2	Herpes Simplex Virus Type 2
ICP	infected cell protein
IFNγ	interferon gamma
K	kidney
kb	kilobases
kg	kilogram
LAP1	latency associated promoter 1

LAP2	latency associated promoter 2
LAT	latency associated transcript
LN_2	liquid nitrogen
LTE	long term expression element
Lv	liver
µL	microliter
µg	microgram
µM	micromolar
mg	milligram
mL	milliliter
mM	millimolar
M	molar
MEM	minimum essential medium
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
MMLvRT	Muloney Murine Leukemia Virus reverse transcriptase
MOI	multiplicity of infection
MOPS	N-morpholino propanesulfonic acid
N	normal
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
NGF	nerve growth factor
nt	nucleotide
P^{32}	phosphorus-32
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
pg	picogram
pol	polymerase
QRT-PCR	quantitative RT-PCR
RCR	reactivation critical region
RNA	ribonucleic acid
R_L	long repeat
R_S	short repeat
RS	rabbit skin cells
RT	reverse transcriptase
RT-PCR	reverse transcriptase- polymerase chain reaction
S^{35}	sulfur-35
SDS	sodium dodecylsulfate
SPF	specific pathogen free
SSC	sodium chloride/ sodium citrate
SV40	simian virus 40
TE	TRIS/ EDTA buffer
TG	trigeminal ganglia
tRNA	transfer RNA

U	unit
U_L	unique long
U_S	unique short

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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May 2004

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Herpes Simplex Virus Type 1 (HSV-1) is a double stranded DNA virus that causes a life-long infection of its host. The infection is characterized by two phases, the acute phase and the latent phase. The virus infects epithelial tissue during the acute infection where it gains access to nerve termini and establishes a latent infection in sensory ganglia neurons. During latency only a single viral transcript is expressed abundantly, the Latency Associated Transcript (LAT). LAT is most abundantly transcribed in neurons, and the 5' portion of the transcript has been implicated in the establishment and reactivation of latent infections. In order to study the regulation of LAT expression in neurons in the absence of viral functions, a transgenic mouse line was created in the C57Bl/6 background containing the region encoding the LAT 5' exon through the 2.0kb intron under the control of its native promoter. Characterization of this transgenic mouse indicates that there is a single copy of the transgene inserted into the mouse genome and LAT expression is abundant in a number of tissues including dorsal root ganglia (DRG),

brain, skin, liver, and kidney. Additionally, *in situ* hybridization indicates that expression of the transgene in the DRG is limited to a subset of cells, similar to what occurs during a natural HSV infection.

During HSV infection of the transgenic mouse, expression of the transgene has no effect on the amount of virus produced during the acute infection in feet, DRG, or spinal cord. Since LAT has been implicated as playing a role in establishment and reactivation of latency, we sought to determine whether expression of the transgene affected the ability of the wild type virus to establish and reactivate from a latent infection. PCR for HSV DNA in DRG detected no difference between transgenic and non-transgenic mice following establishment of HSV latency. Reactivation by explant co-cultivation of latently infected DRG exhibited a similar pattern of reactivation for both transgenic and non-transgenic mice. Taken together, the infection data suggest that LAT is not functioning in trans to regulate the HSV infection. This suggests that LAT acts in *cis* to regulate reactivation.

CHAPTER 1
INTRODUCTION

HSV Clinical Features

Herpes viruses are characterized by their ability to establish a life-long infection of their host with long periods of latency during which the virus exists in ganglionic neurons with only a single transcript detected abundantly. Herpes Simplex Virus Type 1 (HSV-1) infection causes lesions commonly known as cold sores. A large portion of the world population, up to 90% in some areas, has been exposed to HSV-1 by adolescence and produce detectable antibodies to the virus (Roizman and Sears, 1996).

Herpes infections have been described since the days of ancient Greece. Infection with HSV-1 is typically characterized by lesions of the epithelium of the mouth or lips, although it can infect other mucosal areas, such as the eyes. A closely related Herpesvirus, HSV-2, causes the same type of lesions, although they are primarily genital in nature (Roizman and Sears, 1996).

The initial phase of infection or primary infection lasts two to three weeks and is often asymptomatic in young children. Once the lesions have healed, the virus enters a latent state where it exists asymptomatically in sensory nerve ganglia. Latency is interrupted by periods of reactivation, which is the result of stress. During reactivation, lesions can recur at the initial site of infection. The frequency and severity of reactivations vary depending upon the individual, although the duration of reactivation lesions is typically shorter than the primary lesions, and asymptomatic shedding is common (Roizman and Sears, 1996).

HSV infection is particularly dangerous in immunocompromised individuals. Due to the inability to mount an immune response to the virus, these patients may develop a number of more severe symptoms such as keratitus, which can lead to blindness (Whitley, 1990). Newborns exposed to primary infection or reactivating HSV at birth are susceptible to systemic infection that could result in fatal encephalitis (Anderson and Nicholls, 1972).

Current treatments for HSV infection include several nucleoside analogs in either topical or oral forms that reduce the severity of reactivations and shorten the duration of lesions. Vaccines have been developed using peptides, attenuated viruses, and killed viruses, all of which provided limited protection against recurrences. No cure is currently available for HSV infection (Koelle and Corey, 2003).

Immune Response to HSV

The immune response to HSV-1 involves both specific and non-specific mechanisms. In the initial stages of infection, CD4+ cells activate macrophages and produce IFNγ. The activated macrophages produce other cytokines to affect other immune cells. Later in the immune response, CD8+ cytotoxic lymphocytes and antibody producing B cells provide specific immunity that helps to clear virally infected cells (Whitley, 1996).

Like other viruses, HSV has developed methods to evade the immune response. For example, it establishes latency in an immune privileged site – neurons do not normally express MHC class I or II. This site of latency greatly reduces the likelihood of CTL-mediated destruction of the host cell (Ward and Roizman, 1998). An example of the ability of HSV to evade the humoral immune response is gC, an HSV virion

glycoprotein that can bind to the complement factor C3b and limit the induction of the complement cascade (Chen et al., 2003)

In order to determine the role of the immune system in the latent phase of HSV infection, a number of mouse strains deficient in specific aspects of the immune response have been infected. These strains were each compared to the relatively HSV-resistant C57Bl/6 strain which contains a functional immune system. C57Bl/6 mice can be infected with HSV and establish a latent infection but do not develop encephalitis as a result of infection (Lopez, 1975; Kastrukoff et al., 1986). In contrast, SCID mice are highly susceptible to HSV as a result of being deficient in both T and B cells. Even so, it was suggested that HSV has the potential to establish latency at early times post-infection in these mice as defined by the observation of neuronal LAT expression in ganglia 1-2 days post infection (Gesser et al., 1994). Interferon knockout GKO mice exhibited a delay in the peak of viral productive infection, but latency was established at a normal rate and HSV infection was not lethal to these mice, suggesting that interferon stimulation of the immune response is not necessary to resolve the acute infection. The moderately susceptible Balb/c strain resulted in a stronger productive infection than seen in C57Bl/6 mice, but no differences were seen in the relative ability of HSV to establish latent infections in these different mouse strains with known differences in immune backgrounds (Ellison et al., 2000). These studies imply that establishment of latency is the result of a virus – neuron response and not mediated by the immune response since latency was established in all strains tested.

General HSV Characteristics

HSV-1 is the prototype of the alphaherpes virus family causing life-long infection of the host. The virus particle is enveloped with at least eleven glycoproteins present on

the surface. The capsid of the virus is icosahedral in shape and the space between the envelope and the capsid, known as the tegument, contains proteins involved in the initial stages of infection. A diagram of the HSV-1 virion structure in Figure 1-1 illustrates the enveloped capsid with glycoproteins present on the outer surface of the envelope (Roizman and Sears, 1996). Grunewald et al. (2003) were able to use electron microscopy to clarify the HSV virion structure illustrating that the capsid arrangement in the tegument is asymmetrical and that the some 750 glycoprotein spikes present on each virion are arranged in an organized manner on the envelope surface.

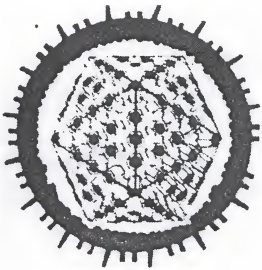

Figure 1-1. Diagram of the HSV-1 virion. HSV is an enveloped virus with an icosahedral capsid and glycoproteins present on the envelope surface (Roizman and Sears, 1996).

The HSV infection is characterized by three phases. During the initial infection or establishment phase, lesions are seen primarily on the oral mucosa. The virus then enters sensory nerve termini and is transported to the sensory ganglia where it establishes a latent infection. The latent phase of infection is characterized by transcriptional activity from a single viral region encoding the latency associated transcripts (LAT), while the

remainder of the genome is silenced. Latent infection is interrupted by periods of reactivation where productive infection resumes and the virus travels through the nerve axons back to the initial site of infection where lesions are seen on the skin surface (Roizman and Sears, 1996).

The HSV-1 genome consists of approximately 152 kilobases (kb) of double-stranded DNA. The genome (Figure 1-2) consists of two unique regions, the unique long (U_L) and the unique short (U_S), each flanked by two repeated regions, the long repeat (R_L) and the short repeat (R_S). The repeat regions are joined together by the "a" sequence, a 500bp highly

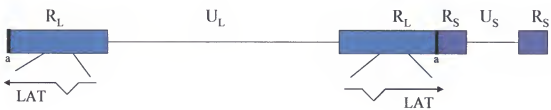

Figure 1-2. Diagram of the HSV-1 genome. HSV possesses a double-stranded DNA genome that is organized into two repeat regions, the repeat long (R_L) and the repeat short (R_S), each flanking unique regions, the unique long (U_L) and the unique short (U_S). The repeat regions are joined together by the "a" sequence. Also shown is the region encoding the latency associated transcripts (LAT).

conserved region of small repetitive elements. HSV genes are identified by their relative time of expression rather than by their position in the genome. The classes of expression are alpha (α), beta (β), and gamma (γ).

During the latent infection, the viral genome associates with cellular histone proteins and circularizes to form an episome, thus existing as a "mini-chromosome" in the infected cell. It has recently been determined that episome formation occurs in the absence of lytic HSV gene products and the ICP0 gene may be involved in the prevention of circularization (Jackson and DeLuca, 2003).

HSV Gene Regulation

Immediate Early Genes

The alpha genes, also known as immediate early, peak in expression 2-4 hours post infection and include infected cell proteins (ICP) 0, 4, 22, 27, and 47. Transcription of the alpha genes is initiated by a complex including the HSV-1 tegument protein VP16 (also known as αTIF) binding to the TAATGARAT octamer located in the immediate early promoters (Mackem and Roizman, 1982). Of the immediate early genes, ICP4 is a strong trans-activator of the early and late viral genes and is essential for viral replication. ICP4 binds to both consensus ATCGTC and non-consensus motifs in several other HSV gene promoters or 5'untranslated regions. The *trans* activation function is promoted by ICP4 binding to DNA as well as the transcription factors TBP and TFIIB (Smith et al., 1993).

While not essential, ICP0 is believed to have multiple functions, including that of a non-specific transactivator (Cai and Schaffer, 1992) and a disruptor of ND10 regions in the nucleus (Davido et al., 2003). The exact purpose of ND10 structures in the nucleus has not been determined, but they may be involved in replication and during the infection a number of HSV proteins interact with ND10. Other studies suggest that ICP0 is involved in reactivation from latency since the latency associated transcripts are expressed from the opposing strand of DNA in the same region as ICP0. Expressing ICP0 in *trans* from an adenovirus vector was sufficient to induce reactivation in latently infected primary trigeminal ganglia cultures (Halford et al., 2001). These data support the theory of LAT acting as a switch to turn on ICP0 during reactivation. The involvement of ICP0 in reactivation will be discussed further in the section on LAT function.

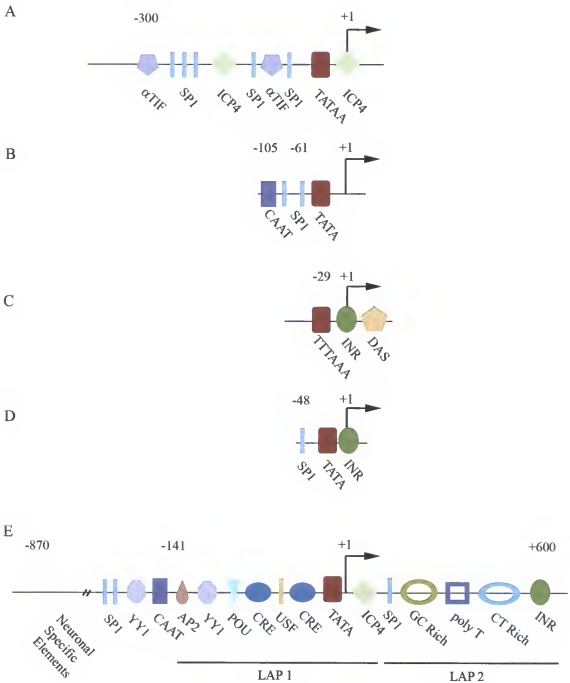

Figure1-3. Regulation of the different HSV gene promoter classes. A. Immediate early genes. B. Early genes. C. Late genes. D. Leaky Late genes. E. LAT gene. Abbrv: INR, initiator element. DAS, downstream activating sequence. LAP1, latency associated promoter 1. LAP2, latency associated promoter 2.

Control of the immediate early genes is primarily under the direction of cellular transcription factors although viral binding sites are also present (Figure 1-3). In addition to a TATA box, immediate early promoters contain several SP1 binding sites and binding sites for the VP16 viral activator protein. The ICP4 binding sites in the promoter allow for down regulation of these genes by ICP4 as the course of infection progresses.

Early Genes

Early β genes include genes involved in viral replication such as the viral polymerase and thymidine kinase. These genes show peak expression 5-7 hours post infection. Despite containing only cellular elements in their promoters including SP1 sites, CAAT and TATA boxes (Figure 1-3), early genes require the viral ICP4 protein to stimulate expression through interaction with the TATA element (reviewed in Weir, 2001).

Late Genes

Structural proteins such as the glycoproteins and tegument proteins make up the group of late γ genes which are expressed only after viral replication has occurred (Roizman and Sears, 1996; Wagner et al., 1998). Late genes are divided into two sub-classes, γ1 and γ2. γ1 genes are leaky lates and can be transcribed in small quantities before viral DNA synthesis has occurred. The γ2 class consists of strictly late genes and dependent upon viral replication.

Promoter sequences for the late genes are much less complicated than the promoters for the two preceding kinetic classes and are limited to a TATA element and a portion of the 5' noncoding region of the transcript acting as an enhancer for high levels of expression (Levine et al., 1990). Expression of some viral genes are required for late

expression, and as an example, a transgenic mouse containing the gC promoter driving β–gal exhibited no expression of β–gal in neuronal or non-neuronal tissues (Loiacono et al., 2002). An example of both late promoter classes is presented in Figure 1-3.

The Latency Associated Transcript

During the latent period of infection, LAT is produced abundantly from the long repeat region of the genome. LAT is the only HSV-1 transcript that has not been classified in one of the classes of genes mentioned above (immediate early, early, or late). The LAT RNA is made as an 8.3-kb primary transcript and is spliced into several smaller RNAs. The most abundant LAT RNA is the 2kb intron that exists as a stable lariat structure with a >24 hour half life (Farrell et al., 1991; Thomas et al., 2002). The stability of the LAT intron may be due to the non-consensus branch point that allows for generation of the lariat structure during splicing (Wu et al., 1998). Additionally, in latently infected trigeminal ganglia a 0.5 kb region is spliced out of the 2 kb intron resulting in a second stable 1.5 kb species (Spivack et al., 1991; Alvira et al., 1999).

There has been no direct evidence that LAT is translated into a protein during the HSV infection despite extensive studies including sequence analysis (Drolet et al., 1998), transient expression assays (Thomas et al., 1999), and site mutagenesis of ATG's (Bloom et al., 1996). Some of these studies were able to generate a LAT protein outside of the context of the natural viral infection, but there is no evidence to date that this protein is expressed during infection (Coffin and Thomas, 1998; Thomas et al., 1999).

Transcription of LAT begins near the TATA box consensus sequence with the promoter extending as much as 870 nucleotides upstream of the transcription start site (Lokensgard et al., 1997). Several cellular regulatory sites have been identified in the

LAT promoter region including cyclic AMP response elements (CRE), Sp1 sites, CAAT box, USF, YY1 and AP-2 (Kenny et al., 1994; Soares et al., 1996; Wagner and Bloom, 1997) as shown in Figure 1-3. The presence of cellular regulatory regions suggests possible cellular control of the LAT promoter which will be examined in the experiments presented in the following chapters.

A second, TATA-less promoter, LAP2 (latency associated promoter 2) has been described in the region 3' of the LAP1 transcription start site (Figure 1-3). This region of DNA contains elements such as a G/C rich segment that are found in housekeeping genes and those genes involved in signal transduction pathways. Transcription from LAP2 is 5-10 fold less abundant than from LAP1 as determined by transient expression with a CAT reporter (Goins et al., 1994). LAP2 is active during the acute phase of the animal infection and also in cell culture of both neuronal (SY5Y) and non-neuronal (CV-1) cells but LAP2 is not active during the latent phase of infection in the absence of LAP1 core promoter elements (Nicosia et al., 1993). The exact transcription start site for LAP2 has yet to be mapped and further studies are necessary to determine the function of transcript(s) derived from the LAP2 promoter. For the remainder of this dissertation the LAT promoter refers to the LAP1 promoter unless otherwise noted.

Previous research has stated that LAT promoter activity is different in different cell types. In addition to expression in neurons during latency, Jarman et al. (1999) reported that LAT is expressed in murine feet during the acute infection following footpad infection using β–gal reporter viruses. The expression was seen two to four days post infection on both the dorsal (infected) and ventral sides of the foot and is in contrast to only low levels of LAT expression observed in non-neuronal cells in culture

(Zwaagstra et al., 1990; Jarman et al., 1999). Additionally, more LAT expression was seen in neuronal cell types (ND7 and C1300) than in non-neuronal rabbit skin cells using *in vitro* infections (Coffin et al., 1998). Further studies with LAT promoter deletions revealed LAT expression differences between neuronal cell cultures and infected dorsal root ganglia neurons (Dobson et al., 1995). This suggests that different neuronal cell types contain different levels or types of transcription factors and that there may be neuronal specific elements in the LAT promoter. *In vivo* two regions in the promoter, -75 to –83 and -212 to –348 relative to the transcription start site, showed increased activity in neuronal cells as opposed to non-neuronal cells (Kenny et al., 1994).

Sensory ganglia contain a variety of cell types, both neuronal and non-neuronal. Margolis et al. (1992) analyzed some neuronal markers of neurons in mouse sensory ganglia that corresponded with either sites of HSV-1 LAT expression, or HSV-1 acute antigen expression and determined that the neuronal population expressing SSEA-3+ as a surface marker exhibited the highest percentage of LAT expression during acute infection in the absence of acute antigen. These differences in LAT promoter activity could be dependent on specific transcription factors present in different cell populations (Dobson et al., 1995; Yang et al., 2000).

Animal Models of HSV Latency and Reactivation

A number of animal model systems are used to study latency and reactivation. In the mouse, infection of the footpad results in latency in dorsal root ganglia (DRG). While not the natural route of infection, the footpad is a large epithelial surface that can support a uniform infection and dissection of infected tissues can be performed easily. One limitation to the mouse model is that HSV reactivation does not result in virus being transported to the primary site of infection as it does in humans. Local reactivation in the

ganglia can be induced however, in one of two ways: co-cult explant of DRG or hyperthermal stress.

The explant model uses the stress caused by dissection of the ganglia from the mouse to initiate reactivation. Dissected ganglia are incubated in tissue culture media and infectious virus is detectable in the media by 2 weeks after explant. Mimicking one of the natural causes of reactivation, fever, the hyperthermia model involves raising the body temperature of the mouse to 43°C for 10 minutes to induce reactivation. While this model initiates the lytic cycle, lesions have not been seen at the initial site of infection but virus can be detected in the sensory ganglion at 24 hours post stress (Sawtell and Thompson, 1992).

Another common model of HSV infection is the rabbit ocular model. Prior to HSV infection rabbit corneas are scarified to allow for a more uniform infection surface. In this model, latency is established in the trigeminal ganglia and HSV can either spontaneously reactivate or be induced to reactivate using iontophoresis of epinephrine to mimic the host stress response (Hill et al., 1986).

There is one non-animal model of reactivation that involves culture of primary neurons in the presence of NGF. For infection, acyclovir (the nucleoside analog used to inhibit HSV lytic genes) and NGF are added to the media so that a quiescent infection is established without killing the neurons. Acyclovir is removed once establishment has taken place and reactivation can be induced by removal of NGF from the media (Colgin et al., 2001). These quiescent cultures are the closest system available to an in-vitro latency model, most tissue culture systems have the ability to support a lytic infection, but not establish latency.

Function of LAT

Establishment and Reactivation

LAT has been linked with a number of different functions during the acute infection, although the most extensive body of data supports a role of LAT in the establishment of and/or reactivation from latency (Wagner, 1991; Roizman and Sears, 1996). The regions of LAT involved in these functions and the others described here are depicted in Figure 1-4.

Using overlapping dermatomes in the mouse, Speck and Simmons (1991) were able to demonstrate the establishment of latency (production of LAT) in the absence of lytic gene production, thus suggesting that lytic and latent pathways can diverge early in

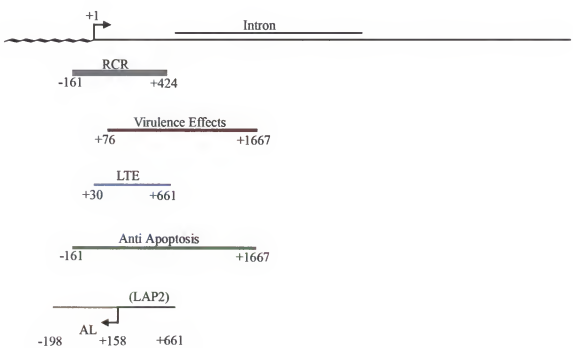

Figure 1-4. Diagram of the function of HSV-1 LAT. Different regions of LAT have been implicated in many functions. The location of these functions are mapped here including the reactivation critical region (RCR), virulence effects, the long term expression element (LTE), and anti-apoptotic region. Also included here is the location of the antisense to LAT transcript, AL.

the infection. Separation of the establishment and reactivation functions was determined by a number of LAT mutants that maintain the ability to establish latency but do not reactivate (Bloom et al., 1994; Bloom et al., 1996; Wang et al., 1997).

These viruses delete either the core promoter sequences of LAT or a region of the LAT 5' exon. Another LAT mutant, containing a large deletion encompassing both the LAT promoter and 827 bp of the 5'exon, established 75% fewer latent infections than its wild type parent virus. It was predicted that this reduction may be due to an increase in neuronal cell death in the ganglia (Perng et al., 1994; Thompson and Sawtell, 2001). Since this virus contains deletions of both LAP1 and LAP2, and is phenotypically distinct from a LAP1 mutant, further studies are needed to determine if the increase in neuronal cell death is related to multiple but genetically separable LAT functions.

Reactivation is typically related to a stress event for the host. In the rabbit eye model, reactivation is seen after iontophoresis of epinephrine onto the eye surface. In the mouse thermal stress model, reactivation of HSV is seen after an increase in basal body temperature. On a cellular level, the mechanism by which stress is translated to reactivation is not known.

Deletion of the LAT core promoter eliminates the ability to reactivate, but viral DNA is still detected in neuronal ganglia. In addition to the LAT promoter, deletions of regions downstream of the transcription start site and extending into the intron have been shown to be important in HSV reactivation. The specific function of this region remains unknown, but deletions of a 348 bp fragment as well as a 371 bp StyI fragment located within the region result in reduced reactivation in the rabbit model (Bloom et al., 1996; Hill et al., 1996; Jarman et al., 2002). Smaller deletions in this region do not affect

reactivation therefore, the act of expressing some LAT is not sufficient for reactivation, and presumably either specific RNA sequences or *cis* acting DNA elements located within the 5' exon are important for reactivation (Bloom et al., 1996; Bhattacharjee et al., 2003). The 5' exon region and the LAT core promoter are referred to as the reactivation critical region (*rcr*).

Since expression of RNA itself is not sufficient for reactivation, other mechanisms of LAT action have been proposed. The regulation of reactivation and/or establishment may be at the DNA level. Many cellular genes use methylation of CpG islands as an epigenetic method of regulating transcription. In these genes, a methyl group is added to the cytosine of the DNA at regions to be either silenced or transcribed. Previous studies determined that methylation was not present in the HSV genome as a whole, but did not look at specific promoter regions of the genome. Using bisulfite analysis Kubat et al. (2004) determined that there is no pattern of methylation as a form of regulating HSV latent transcription.

These data suggest that instead of methylation, the virus uses the DNA chromatin structure and its association with specific histone proteins as a means of regulation. Further studies indicate that there is a difference in the chromatin acetylation pattern during latency when comparing the LAT promoter to other acute HSV promoters (Kubat et al., 2004).

Virulence

Several LAT deletion mutants have an effect on virulence in either mice, rabbits, or both experimental systems. The dLAT1.5 virus contains a 5'exon deletion of ~1600bp and is increased for virulence in mice. Increased virulence in rabbits and decreased virulence in mice were seen with LAT2.9A which contains only a 371 bp deletion in the

5' LAT exon. This virus was also reduced for spontaneous reactivation in the rabbit (Perng et al., 1999).

Other mutants deleting either a portion of the 5'exon (Δ307) or the 3' portion of the intron (Δ480) exhibit decreased virulence (Gary et al., in preparation). These experiments on the whole indicate that there are differences in the infection between mice and rabbits since the deletion viruses produce different effects dependent on the model system used. Further studies are necessary to determine the mechanism employed by these viruses in both the mouse and rabbit models.

Enhancer

A number of studies have demonstrated that the LAT core promoter, by itself, is not sufficient to direct long-term expression (Margolis et al., 1993). The long-term expression element (LTE) has been described as the region of the 5' exon from PstI to the splice junction (Berthomme et al., 2001). Analysis using reporter constructs containing this region downstream of the core LAT promoter continued to express β–gal at 40 days post infection while the control (containing just the LAT core promoter) had no expression at 28 days post infection (Lokensgard et al., 1997). Transient expression experiments containing the LTE showed that the LTE region can activate the HSV-1 thymidine kinase promoter in both neuronal and non-neuronal cells (Berthomme et al., 2000). These data suggest that the LTE contains an enhancer element and functions to promote long term LAT expression.

Neuronal Survival

LAT has also been implicated as a suppressor of apoptosis. A large LAT deletion (-161 to +1667 relative to the LAT transcription start site) demonstrated increased

apoptosis by both TUNEL and PARP assays (Perng et al., 2000). Ahmed, et al. (2002) stated that plasmids lacking the sequences in the 5' LAT exon were least efficient in blocking apoptosis, and that this region appears to contribute to cell survival. This is the same region that contributes to the virulence and reactivation phenotypes mentioned above (Figure 1-4). Additionally, Thompson and Sawtell (2001) have used LAT null viruses to examine cell survival during infection. They showed that 75% less establishment of latency occurred in the LAT null virus and this was accompanied by a large amount of neuronal death. They propose that one function of LAT is to protect sensory neurons from death and enhance the establishment of latency.

Other Possible LAT Functions

Since LAT is transcribed antisense to ICP0 and the ICP0 gene is one of the first promoters activated in the lytic cascade, it was believed that LAT could have an antisense effect on ICP0 expression. A portion of the 2 kb LAT intron overlaps with the ICP0 transcript. In clonal cell lines containing these regions of DNA, Burton et al. did not find an antisense effect with ICP0 when expressing LAT in *trans* in non-neuronal cells (Burton et al., 2003). This does not mean that LAT cannot act in *cis* to affect the ICP0 region of transcription, perhaps acting on the DNA itself or in a structural manner to open or close the region of DNA.

Another antisense function involves the transcript AL (antisense to LAT) which is transcribed 6-8 hours post infection in PC-12 cells. AL is located in the 5'exon and core promoter region of LAT (Figure 1-4), and may have an opposing or balancing function with LAT although more research is needed to specifically define AL and its function in the HSV infection (Perng et al., 2002). To date the AL transcript has only been detected

in productively infected PC-12 cells in culture using RT-PCR, and can not been detected *in vivo*.

LAT also has a repressive function. A LAT mutant which had reduced LAT expression exhibited an increase in the amount of ICP4 and thymidine kinase expression compared to wild type infection (Chen et al., 1997). A possible mechanism for this repressive function may be explained by regions of sequence homology to *Xist* suggesting that LAT may function to "paint" the HSV chromosome to silence it similar to *Xist* painting the inactive X chromosome in mammalian cells (Bloom et al., 1996).

The HSV LAT region encompasses a number of transcripts present on both strands of DNA. The various functions for LAT presented here may be important at different times during the course of HSV infection, and some may work in combination with other viral functions making them difficult to phenotypically dissect in the context of the virus. It is also possible that some of these functions are not manifest during the *in vivo* infection since they have been discovered and tested in cell culture systems only. This complexity of dissecting LAT functions provides the basis for generating a transgenic mouse containing this region of LAT DNA to further study its functions in the absence of the HSV genome and its contributed *cis* and *trans* acting viral functions.

Use of Transgenics to Study Disease

Expression of Acute Genes in Transgenic Mice

Transgenic technology has been used to study the *roles* of genes out of their native context. To generate a transgenic animal, the gene of interest is injected into the single cell fertilized oocyte nucleus typically of mice, although other species have been used as well. By injecting the single cell, the transgene is able to be integrated and replicated with each cell division and is then present in every cell of the animal. The

expression of the transgene is generally dependent upon the promoter used to drive the inserted gene.

In the case of HSV-1, transgenic mice have been created to study regulation of the different classes of HSV genes. These mice each contain the HSV promoter in question driving a *lacZ* reporter. An ICP4 transgenic mouse expressed such a reporter under the control of the ICP4 promoter primarily in neuronal tissues, with lower levels of expression in trigeminal ganglia and retinas and high levels of expression in brain regions and the dorsal horn of the spinal cord (Mitchell, 1995). Additionally, ICP0 and ICP27 transgenic mice demonstrated similar expression in neuronal tissues (Loiacono et al., 2002). In addition the ICP0 and ICP4 transgenic mice were shown to differentially express the reporter based on the age of the mice. ICP4 transgenic mice had approximately 100-fold greater expression in newborn mice compared to adults. Differential expression of ICP0 was the reverse of the expression seen with ICP4, with expression increasing with age. These findings indicate that although HSV immediate early promoters contain similar regulatory elements, they are regulated differently by cellular factors in the absence of other HSV proteins. In contrast to the findings with the IE promoters, both neuronal and non-neuronal cells were negative for expression in a gC transgenic mouse, illustrating that HSV late promoters require other viral functions for their expression (Mitchell, 1995; Loiacono et al., 2002).

Expression of LAT in Transgenics

Previously a LAT transgenic mouse had been created to specifically study the splicing of the 1.5 kb LAT out of the 2 kb LAT intron. This mouse contains the 2 kb LAT of HSV-1 under control of a CMV promoter. Studies have determined that the splicing event of the 1.5 kb species is more efficient in neural tissues than non-neural

tissues and suggested that the LAT transgenic mouse reactivates more efficiently than non transgenic littermates (Mador et al., 2003). One of the drawbacks of this transgenic mouse model is that the CMV promoter was used instead of the native LAT promoter. The CMV promoter is a strong constitutive promoter that has activity in all cell types. Thus, while the splicing event may be specific to LAT, expression patterns may permit splicing in cell types that are non-permissive for the latent HSV infection.

Using the LAT region from HSV-2, Wang et al. (2001) studied establishment and reactivation in a transgenic mouse. HSV-2 LAT was driven by its native promoter and had expression to high levels in neural tissues as well as some non-neural tissues determined by northern blots of tissue RNA. The expression in non-neural tissues was not expected, but not surprising since Jarman et al. had shown LAT expression in the feet during acute HSV infection (Jarman et al., 1999). In these mice expressing LAT in *trans* had no effect on the HSV-2 infection at the establishment or reactivation level. Since there are a number of differences in tropism between HSV-1 and HSV-2, and because the HSV-2 LAT intron is processed differently than that of HSV-1, the construction and analysis of an HSV-1 transgenic mouse model expressing the HSV-1 LAT from its native promoter was desirable and the focus of this dissertation.

Hypotheses to be Tested Using the Transgenic Approach

Generation of Transgenic Mouse

In the studies presented here, we have generated a LAT transgenic mouse from HSV-1 strain 17+. This mouse line uses the native HSV-1 LAT promoter and contains the region encoding the LAT transcript 5' exon and 2 kb intron. The rationale for using these regions will be discussed further in Chapter 2. Initial characterization of the mouse

includes determination of the transgene copy number, mapping the transgene insert and gross tissue-level expression studies.

Expression of LAT in *trans*

Since the LAT transgene is being controlled by the native LAT promoter and the region of the promoter shown to contain neuronal specific elements was included in the sequence inserted, it was hypothesized that LAT would be expressed in neuronal tissues. Data from the HSV-2 transgenic mouse suggests that LAT can be expressed in non-neuronal cells as well, although the expression was not quantitated (Wang et al., 2001). In this study, quantitative RT-PCR was employed to determine if neuronal cells could more efficiently produce the LAT transcript than non-neuronal cells. In addition to quantitative RT-PCR of RNA extracted from whole tissues, *in situ* hybridization techniques were used to determine if expression is from all cells or a subset of cells.

Infections

Effect of LAT on the course of infection

It is known that in the context of the HSV genome LAT is expressed in neuronal cells during latency. It was possible that expressing LAT prior to the infection, *in trans*, could have an effect on the course of infection, such as altering the establishment of latency, or the ability of the virus to reactivate from latency. To study these effects, transgenic mice and their non transgenic littermates were infected with wild type 17+ HSV-1. Similar to the observations made in the case of the HSV-2 mouse, we hypothesized that expressing LAT in *trans* would not alter the HSV infection, and that during a natural HSV-1 infection, LAT functions in *cis* on the HSV genome.

Restoration of virulence by expressing LAT in *trans*

One of the functions attributed to LAT is a change in virulence (Gary et al., in preparation; Perng et al., 1999). By infecting LAT transgenic mice with a LAT mutant that is reduced in virulence, we hypothesized that the expression of the transgene containing the region deleted in the mutant could restore wild type virulence level if the function can act in *trans*. In this case, virulence was measured as a function of virus titers reaching the DRG and assayed by titering the amount of infectious virus present in feet and DRG during the acute infection following footpad inoculation.

In summary, HSV-1 LAT is a complex region with a number of functions attributed to it. Generation of a transgenic mouse containing this region can be used to further define some of these functions a well as to determine which functions are trans-acting and can be attributed to the expression of the RNA.

CHAPTER 2
GENERATION OF A TRANSGENIC MOUSE EXPRESSING A PORTION OF THE HSV-1 LATENCY ASSOCIATED TRANSCRIPT

Overview

The latency-associated transcript (LAT) of HSV-1 has been implicated as playing a role in a number of functions related to the viral infection. The mechanisms of these functions and the regulation of the LAT transcript have not been determined. To examine the regulation of the LAT in cells, we have generated a transgenic mouse containing the LAT inserted into the mouse genome. This therefore has allowed us to study the LAT's function outside its normal context of the HSV genome.

Transgenic technology was first used in 1980 to inject HSV and SV40 viral plasmid DNA into a fertilized mouse pronucleus (Gordon et al., 1980); and has since expanded to include transgenic animals for a number of human disease models and viral gene models (Nomura, 1997). The use of mice for transgene studies is ideal because of the knowledge of mouse genetics and the number of different genetic strains available.

Although other transgenic mice containing HSV LATs have been generated, they did not contain the native promoter sequence or the LAT from HSV-1 (Wang et al., 2001; Mador et al., 2003). Thus, the HSV-1 LAT transgenic mouse described here is a novel model system because it allows for LAT to be regulated by its native promoter. This mouse has provided a means for studying a number of the proposed LAT functions including reactivation, virulence, and neuronal survival.

In addition to determining whether a transgenic mouse line expresses its transgene, characterization of any new transgenic mouse line routinely requires determining the copy number and the integration site of the transgene. The location of the transgene in the genome may effect transcription of the transgene by position effects. If the transgene inserts into a silenced region of the genome, it may not be expressed or expressed at low levels due to the regulation of the surrounding region of DNA (Sippel et al., 1997).

The goal of this dissertation was to generate a HSV-1 LAT transgenic mouse to study LAT regulation by cellular factors in the absence of viral *cis* and *trans* factors. This chapter describes the construction, breeding strategy, and the initial characterization of the transgene in the HSV-1 LAT transgenic mouse line.

Materials and Methods

Plasmid Used to Generate the Transgenic Mouse

The pLAT/LAT plasmid was generated to construct the transgene insert. A portion of the HSV-1 strain 17+ latency associated transcript (LAT), including the DraI site (corresponding to HSV-1 genome base pair 116,516) to the AatII site (corresponding to HSV-1 genome base pair 121,549), was ligated into a pBluescript backbone at the SmaI site. The SV40 poly A sequence from pNSE-Ex4 (a gift from G. Rall, The Fox Chase Cancer Center) was removed using EcoRI and inserted at the XbaI site into the pBluescript plasmid containing the LAT sequence. Figure 2-1 diagrams the region of the LAT gene used in constructing the transgene as well as its location in the HSV genome. The complete plasmid sequence and map is presented in Appendix A.

Screening for Founders Containing the LAT Insert

Fertilized oocytes were obtained from C3H/HeJ mice. Purification of the transgene, preparation of mice, microinjection into fertilized oocytes and embryo implants were carried out by Dr. Glenn Rall at Fox Chase Cancer Center as described (Hogan et al., 1986; Rall et al., 1995).

DNA from the founder generation (F1) of mice was obtained by clipping a 1 cm portion of the tail from anesthesized mice. Tail clips were incubated overnight at 50°C in 0.5 mL STE buffer (50 mM TRIS pH 8, 100 mM NaCl, 1mM EDTA, 1% SDS) and 1 mg Proteinase K. After incubation, hair and bone were removed by centrifugation at 20,000 x *g* and the supernatant transferred to a new centrifuge tube. DNA was extracted with equal amounts of phenol and sevag (1:24 ratio of isoamyl alcohol and chloroform) followed by extraction with sevag alone. DNA was precipitated with 100% ethanol. The DNA pellet was resuspended in TE (10mM TRIS, 1mM EDTA) pH 8 and quantitated by spectrophotometry at A_{260}. The founder generation was screened by Glenn Rall using slot blot hybridization analysis. Briefly, 10 μg of DNA per slot of a slot blot apparatus was applied to a nylon membrane for hybridization as described by Rall et al. (1995). The membrane was probed with a ^{32}P-labeled DNA fragment from either the SV40 poly A sequence in the pNSE-Ex4 plasmid or a portion of the LAT transgenic insert fragment corresponding to 119,193 to 120,090 nucleotides (nt) of the HSV-1 strain 17+ genome. The nt determinations for all genomic HSV sequences presented in this dissertation are according to McGeoch numbering (Perry and McGeoch, 1988).

Genotyping of subsequent generations was determined by PCR analysis. DNA was prepared from tail clips as described above. pLAT/LAT plasmid DNA was used as a

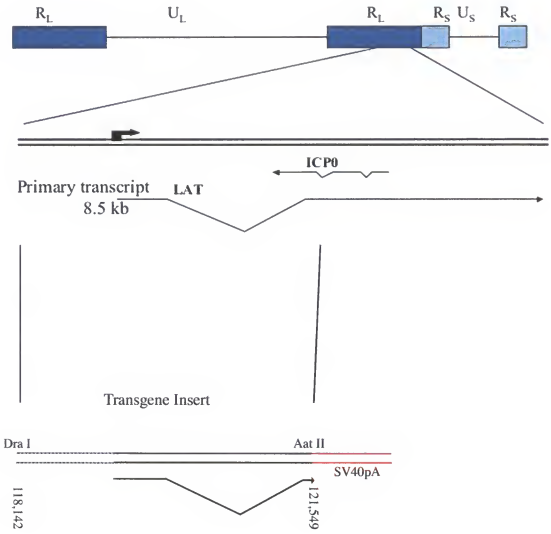

Figure 2-1. Diagram of the LAT Transgene Insert. Shown is the HSV-1 genome with the LAT region expanded to include the location of the LAT and ICP0 genes. LAT is further expanded to illustrate the exact region included in generating the transgenic mouse. For the transgene insert the promoter is illustrated as a dotted line. A SV40 poly A sequence, shown here in red, was added to stabilize the expressed RNA.

positive control for all genotyping PCR reactions. PCR primers were located in the 5' exon region of LAT corresponding to nt 118,888 to 119,037 of the HSV-1 genome (forward: 5'CGG CGA CAT CCT CCC CCT AAG C3' and reverse: 5'GAC AGA CGA ACG AAA CAT TCC G3'). Each reaction contained 200 ng of tail DNA, 0.5 μM of

each primer, 1.5 mM Tris pH 8.8, 16.6 mM ammonium sulfate, 2 mM magnesium chloride, 0.17 mg bovine serum albumin, 1.25 mM each dGTP, dCTP, dATP, dTTP and 2.5 U Taq polymerase (Perkin Elmer). PCR was performed using a Ericomp thermalcycler (San Diego, CA) using the following conditions: one cycle 3 minutes 94°C, 3 minutes 55°C, 3 minutes 72°C followed by 30 cycles 1 minute 94°C, 1 minute 55°C, 1 minute 72°C.

PCR products were viewed on a 7% polyacrylamide gel using SYBR green (Molecular Probes, Invitrogen) and a Storm Phosphoimager. The intensity of the tail DNA amplification products were compared to PCR's of dilutions of the positive control pLAT/LAT plasmid to determine the transgenic genotype by quantitating the intensity of the bands using Image Quant software.

Breeding of Transgenic Mice

All mice were maintained under specific pathogen free (SPF) conditions with access to food and water at will. Each cage contained corn cob bedding and cotton nestlets. Cage bedding was replaced bi-weekly.

The positive founder mouse was backcrossed with a C57Bl/6 mouse and the resulting litter screened by hybridization for presence of the LAT transgene. The F2 generation transgenic positive mice were again backcrossed in the C57Bl/6 background. All breeder animals were at least 8 weeks old. Initial C57Bl/6 breeders were obtained from Harlan. A small C57Bl/6 colony was then maintained to provide C57Bl/6 breeders. Mice were maintained as for the transgenic colony although only a single breeder pair was needed and genotyping was not necessary for these mice.

Transgenic breeder cages contained one male with up to three females. Males were kept in the same cage with the breeder females for one week to ensure pregnancy. At approximately 18 days post breeding, females were separated into individual cages to deliver their litters. Pregnant females and newborns were monitored at least twice daily for difficulty with delivery and/or nurturing. Pups remained with their mother for three weeks until they were weaned and sex-separated. Genotyping of the weanlings was performed approximately one week after weaning.

Backcrosses continued until obtaining the F10 generation at which time the transgenic background was considered to be genetically C57Bl/6 and were crossed with F10 littermates to generate homozygous transgenics. All mice used in the studies described here are heterozygous for the LAT transgene and were of at least the F4 generation.

Determination of Transgene Copy Number

Slot blot hybridization

Slot blot hybridization was used to determine transgene copy number. DNA from tail clips of weanlings was isolated and quantitated as described above for genotyping. For each sample 10 μg DNA, 15 μL 3 M NaOH, and 130 μL TE in a final volume of 175 μL was vortexed and incubated at 65°C for 30 minutes. During the incubation, the slot blot apparatus (Gibco BRL) was loaded with Zeta Probe membrane (Bio Rad) pre-wet in water followed by 15x SSC. Incubated samples were ice-quenched and 165 μL of 2 M ammonium acetate added just prior to loading into the blot apparatus. Vacuum was applied after all the samples had been loaded.

Prior to hybridization the blot was baked for two hours in a vacuum oven at 80°C. Pre-hybridization and hybridization were carried out at 65°C in 20 mL of hybridization buffer (5x SCC, 5x Denhardt's solution, 1% SDS) in a sealed bag with each incubation lasting overnight. ATD19 probe (nt 119,664 to 119,972 bp of the HSV genome) labeled with ^{32}P was added to the buffer after the first overnight incubation. The labeled blot was washed twice for 15 minutes each at room temperature with 0.3 M NaCl, 0.06 M Tris pH 8.0, 0.002 M EDTA followed by two washes for 15 minutes each at 65°C with 0.3 M NaCl, 0.06 M Tris pH 8.0, 0.002 M EDTA, 4% SDS. After washing the blot was dried on Whatman paper and exposed to a phosphor screen overnight. The intensity of the radiolabled bands was detected on a STORM phosphorimager and quantitated using image quant software.

Real time PCR

To confirm and further quantitate the transgene copy number, a comparison between the cellular *Xist* gene and LAT transgene was made using real time PCR. Reactions were performed using primers specific for the 5' exon of the LAT and *Xist*. Primer and probe sequences were 5'LAT forward: 5'GGC TCC ATC GCC TTT CCT, 5'LAT reverse: AAG GGA GGG AGG AGG GTA CTG, 5' LAT probe: 5'TCT CGC TTC TCC CC, *Xist* forward: 5'GCT CTT AAA CTG AGT GGG TGT TCA, *Xist* reverse: 5'GTA TCA CGC AGA AGC CAT AAT GG, *Xist* probe: 5'ACG CGG GCT CTC CA. PCRs were performed on an ABI Prism 7700 thermal cycler (Applied Biosystems) located in the ICBR protein core at the University of Florida. Ten-fold dilutions of the LAT transgenic plasmid (pLAT/LAT) corresponding to 10^4 to 10^1 copies were used to generate a standard curve. For the *Xist* cellular control, a standard curve of the pB1/B10

plasmid, a generous gift of Dr. Jeannie Lee (Shibata and Lee, 2003), was diluted 10 fold between 10^5 and 10^1. Samples were run in triplicate. Each reaction contained 10 ng tail DNA, 0.33 μL 60x Assay Mix (primer/probe set), 10 μL Taqman Universal PCR Mix (Applied Biosystems part #430437) in a final volume of 20 μL. PCR was performed in 96 well plates under the following conditions: 1 cycle 2 minutes 50°C, 1 cycle 10 minutes 95°C, 40 cycles 15 seconds 95°C 1 minute 60°C.

Mapping of Transgene Insert

Mapping of the LAT DNA inserted into the transgenic mouse was determined using both conventional and real time PCR. For conventional PCR, reactions contained 600 ng each of forward and reverse primers, 20 μL Hot Master PCR mix (Brinkman Eppendorf) and 200 ng tail DNA in a 50 μL final reaction volume. Control PCR reactions contained 600 ng each of forward and reverse primers, 20 μL Hot Master PCR mix and 0.5 ng pLAT/LAT plasmid DNA in a final reaction volume of 50 μL. Primer sequences, genome locations and optimal conditions for all primer sets used are found in Appendix B.

Real time PCR was used for two regions of the transgene, one in the promoter and one in the 5' exon. Primer and probe sequences for these reactions are found in Appendix B. All real time reactions were performed on an ABI Prism 7700 thermal cycler (Applied Biosystems) located in the ICBR protein core at the University of Florida. Samples were run in triplicate. For each reaction, 50 ng of tail DNA was added to 0.33 μL 60x Assay Mix (primer/probe set) or 1 μL 20x Assay Mix, and 10 μL Taqman Universal PCR Mix (Applied Biosystems part #430437) in a final volume of 20 μL. Control reactions used 1 ng of pLAT/LAT plasmid in place of the tail DNA. PCR was performed in 96 well plates

under the following conditions: 1 cycle 2 mintues 50°C, 1 cycle 10 minutes 95°C, 40 cycles 15 seconds 95°C 1 minute 60°C.

Northern Blot of Transgenic RNA

Harvesting of transgenic tissues

Transgenic mice were euthanized with halothane and brain, DRG, liver, kidney, and foot were dissected. Tissues were snap frozen in liquid nitrogen and stored at -80°C until processed for RNA.

Isolation of RNA from tissues

Tissues were homogenized in 400 μL Trizol reagent (Invitrogen) using Kontes glass tissue grinders (Fisher) or a ceramic mortar and pestle for larger tissues (i.e. skin, feet, liver). Sterile sand was added to the mortar to aid in grinding of the feet. Grinders were rinsed twice with 400 μL Trizol each and this rinse solution added to the ground tissue fraction. After 5 minutes at room temperature, 240 μL of chloroform was added and homogenates vortexed 15 seconds followed by a 5 minute incubation at room temperature. Tissue homogenates were centrifuged at 9,000 x *g* for 15 minutes at 4°C and the aqueous phase containing the RNA was removed to a separate tube. The bottom Trizol layer was stored at -80°C for subsequent back-extraction of DNA, if necessary.

RNA was precipitated with 500 μL of isopropanol at room temperature for 10 mintues followed by centrifugation at 12,000 x *g* for 10 minutes at 4°C. RNA pellets were washed with 1 mL 70% ethanol and centrifuged 5 minutes at 4°C, 7,500 x *g*. The resulting pellet was air dried briefly and resuspended in 200 μL diethyl pyrocarbonate (DEPC, Sigma)-treated water.

Preparation of formaldehyde-agarose gels and blotting of RNA

To prepare the gel for RNA, a slurry of 1 gram of agarose was prepared in 75 mL of sterile DEPC-treated water and microwaved to melt the agarose. The melted agarose was then cooled to approximately 60°C and, just prior to pouring the gel, 20 mL of 5X 3-(N-morpholino) propanesulfonic acid (MOPS) and 5.3 mL of a 37% formaldehyde solution (v/v) was added and mixed gently to prevent bubbles from forming in the gel. The gel was allowed to harden for at least one hour before loading the RNA.

For each RNA sample, 5 μg of RNA was mixed with 15.5 mL FFLB (10 parts formamide, 3.5 parts 37% formaldehyde, and 2 parts 5x MOPS) in a total volume of 20 μL and incubated for 15 minutes at 65°C. Samples were snap-cooled on ice prior to loading on the gel, and 2 μL of RNA dyes (50% glycerol, 1mM EDTA, 0.25% bromophenol blue, 300 μg/μl ethidium bromide) were added to each sample for loading. Running buffer consisted of 160 mL 5x MOPS, 43 mL 37% formaldehyde q.s. to 800 mL with DEPC-treated water. The RNA was electrophoresed at 85 volts for approximately 3 hours. The dye front ran approximately 9 cm from the wells on a 14 cm gel. RNA was viewed using ultraviolet light and photographed with a digital camera (Kodak Photo Documentation System).

Transfer of RNA to membrane

The RNA was transferred onto to Zeta Probe Nylon Membrane (Bio Rad) according to the manufacturer's directions. The gel was rinsed briefly in water and transfer set up using Whatman paper as the wicking for capillary transfer. In addition to the membrane located on top of the gel, four layers of whatman paper and 2 inches of paper towels were included. For the transfer solution, 10X SSC was allowed to absorb

from the reservoir through the wicking papers at room temperature overnight. The transferred membrane was rinsed in Nanopure water and baked at 80°C in a vacuum oven for 30 minutes to crosslink the RNA to the membrane.

Hybridization of northern blot

ATD19 probe (corresponding to nt 119,664 to 119,972 from the HSV genome) was random hexamer primed (Random Labeling Kit, Roche) and labeled with ^{32}P dCTP. The blot was pre-hybridized for 3 hours at 42°C in 20 mL FPH buffer (5X SSC, 5X Denhardt's solution, 50% Formamide, 1% SDS) in a sealed bag. Labeled probe was added through a small cut in the corner of the bag and resealed. The hybridization was incubated overnight at 42°C.

The Northern blot was washed twice at room temperature with 50 mL 2X SSC/0.1% SDS for 5 minutes each followed by two washes in 0.2X SSC/0.1% SDS for 5 minutes each at room temperature. The blot was dried briefly on Whatman paper and exposed to a phosphor screen for 5 hours. A STORM phosphorimager was used to scan the blot and the intensity of bands measured by Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Results

Determination of the Number of Copies of the LAT Transgene

When generating transgenic animals, it is common for multiple copies of the transgenic insert to be integrated into the animal genome (Ellis et al., 1997). There are a number of ways to determine the number of integrated copies, including hybridization and PCR. Initially, we used slot blot hybridization to quantitate the copies of LAT transgene present in these mice. In this case, the LAT hybridization signal from a known

quantity of transgenic-positive tail DNA was compared to the hybridization signal generated from a known number of copies of LAT plasmid DNA spiked into a negative tail DNA sample to represent increasing copies per cell of the transgene. Comparison of the transgenic-positive and transgenic-negative mouse DNA to the control samples indicated that there was a low number of copies present in the transgenic mouse (figure 2-2). The difficulty in distinguishing between the positive and negative samples may have been due to an error in dilution of the standards since this involved a spectrophormetrically determined quantity.

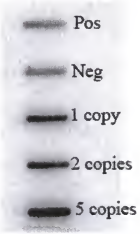

Figure 2-2. HSV transgene copy number determination by slot blot hybridization analysis. Tail DNA from a transgenic (pos) and non-transgenic (neg) mouse was compared to known copies of a plasmid containing the transgene, in the background of tail DNA from a non-transgenic mouse.

Hybridization is not a reliably quantitative method, particularly with low copy numbers, thus to more accurately evaluate the number of LAT transgenes inserted we switched to real time PCR. For this system of analysis, a cellular gene of known copy number, *Xist*, was compared to the number of copies of LAT in transgenic tail DNA. Since *Xist* is located on the X chromosome, the sex of the animal was taken into consideration and the copies of *Xist* for female mice was divided by 2 to standardize

samples to compare the LAT transgene to a single copy of the cellular control for both male and female samples. Table 2-1 contains the PCR data for the cellular and LAT PCR reactions. Although the copy number determined by this analysis is less than one, when taking into account the error of the samples, and the fact that there has to be an integer number of copies, the most likely interpretation is that there is only a single copy of LAT present in these mice.

TABLE 2-1 Determination of transgenic copy number by real-time PCR.

	Xist Copies[1]	**LAT copies**	**LAT/Xist**
1	3.03×10^3	1.28×10^3	0.422 +/- 0.109
2	1.56×10^3	5.43×10^2	0.348 +/- 0.209
3	1.47×10^3	4.30×10^2	0.293 +/- 0.193
4	2.69×10^3	2.80×10^3	1.041 +/- 0.306
5	3.39×10^3	5.83×10^3	1.720 +/- 0.385
Average			**0.765 +/- 0.240**

[1] +/- 730 copies

While mosaicism is possible in up to 30% of transgene insertions (Wilkie et al., 1986), the genetic inheritance from the breeding of these mice does not suggest that a mosaic is present. In a mosaic animal, the transgene inserted into the genome after the first cell division and is therefore not present in the genome of each cell in the animal. If this were the case, only a portion of the germ cells would contain the transgene and thus less than 50% of the offspring of a transgenic and wild type mating would carry the transgene. With almost 500 offspring from transgenic and wild type matings to date, we have not seen evidence consistent with mosaicism in the LAT transgenic mouse.

Mapping of the Transgene Insert

Generation of the transgenic founder mouse was more difficult than usual, requiring three separate sets of injections to obtain a single founder. Screening for the

founder mouse determined that the SV40 poly A signal has been deleted, thus the founder was LAT positive and SV40 negative (G. Rall, personal communication). PCR analysis of DNA from transgenic mice was used to confirm the extent of the LAT transgenic insert present in the transgenic line. As described in the materials and methods, both conventional and real time PCR were used to map a large portion of the transgene. Figure 2-3 illustrates the location of primer sets used to map the transgene, and indicates those that were positive for presence of the transgene. The regions analyzed by conventional PCR are represented by black arrows, while the regions analyzed by real time PCR are shown in red. Also shown are the locations of two probes, ATD17 and ATD19 (shown in blue) which were used in hybridization analyses described later that also confirmed the presence of the regions of the LAT in the transgenic mouse.

The PCR products resulting from the conventional PCR reactions (figure 2-4) illustrate bands of the indicated sizes with both pLAT/LAT plasmid and mouse tail DNA. One additional primer set AG29 and AG31 failed to detect the corresponding LAT sequences in the DNA from the transgenic mouse. The location of these primers is represented in green, and corresponds to the 3' end of the transgene insert (figure 2-3). Since this primer pair (AG29 &31) has failed to detect a product from transgenic DNA it is believed that a portion of the 3' end of the transgene has been deleted in addition to the SV40 poly A signal.

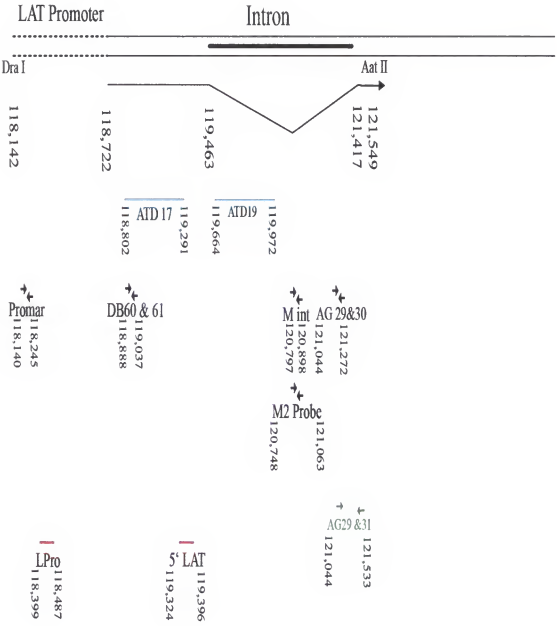

Figure 2-3. Mapping of the LAT transgene in the LAT transgenic mouse. The blue lines represent the location of hybridization probes positive with the indicated LAT regions. Black arrows depict primers used for conventional PCR analysis positive for the transgene, and primers used for real time PCR analyses are shown in red. Illustrated in green are conventional PCR primers that were positive in reactions with the transgenic plasmid but not in reactions containing DNA from the transgenic mouse suggesting that this region has been deleted.

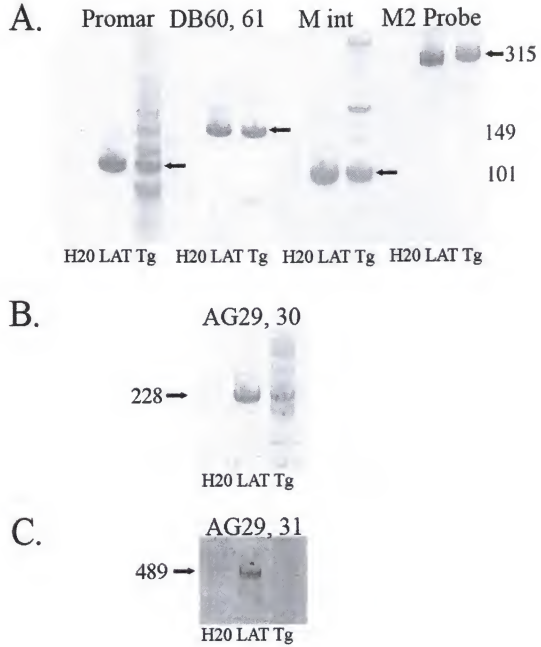

Figure 2-4. PCR mapping the transgene insert. A. Four primer sets, Promar, DB60&61, M int, and M2 Probe, all present in both the LAT plasmid (LAT) and transgenic mouse (Tg). H_2O lanes are no template control. B. Primer set AG 29&30 present in both LAT plasmid and transgenic mouse. C. Primer set AG29&31 present in LAT plasmid but not transgenic mouse. The location of the primer sets in the HSV genome are diagrammed in figure 2-3.

Determination of Transgene Expression by Northern Blot Analysis

Initial expression studies to determine if LAT was active in the transgenic mouse line were carried out using a Northern blot and probing for the LAT intron which typically accumulates to high levels in ganglia of infected mice. Since the transgene is present in all cells of the mouse, neural and non-neural tissues were analyzed to see if expression or accumulation of this intron was different in the various types of tissue. DRG that were latently infected with HSV were used in this experiment as controls for both the presence and size of the stable intron.

Figure 2-5b demonstrates that the LAT intron is accumulating in the DRG of transgenic mice but not in the other tissues tested (Liver, Kidney, Brain, Feet). The RNA gel (figure 2-5a), when photographed using ultraviolet light indicated that there was RNA present in each of the wells and that there was more RNA present in the Kidney and Brain samples than the DRG sample. Thus, the lack of hybridization in the samples was not due to the absence of RNA. Lack of intron accumulation in tissues other than the DRG does not mean that LAT is not being expressed in those tissues. In the tissues not accumulating LAT intron, splicing may be inefficient or the intron maybe destabilized. The presence of a higher molecular weight band in the brain sample (figure 2-5b) may support these theories but further studies are necessary to confirm the mechanism involved in the lack of intron accumulation. The size of the LAT intron band (relative to the sizes of the 28s and 18s RNA bands) when compared to the controls (DRG compared to K6) indicates that the intron from the transgenic mouse is complete, measuring to approximately 1.9 kb.

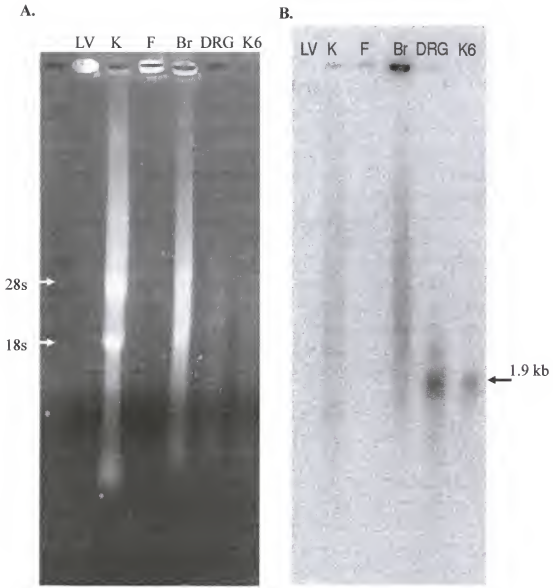

Figure 2-5. Expression of the LAT transgene. A. Agarose gel of total RNA from transgenic and HSV-1 infected mice. In the transgenic total RNA was isolated from liver (LV), kidney (K), foot (F), brain (BR), and dorsal root ganglia (DRG). Control RNA from HSV infected mice (K6). The location of 18s and 28s RNA is marked on the gel. B. Northern blot using a probe for the LAT 2kb intron. Accumulation is seen only in the infected DRG and the transgenic DRG. A larger molecular weight band in the brain suggests that there may be a splicing difference between different types of tissue.

Discussion

Unlike typical transgenic inserts, the LAT founder mouse was particularly difficult to produce and required three separate injections. When the founder mouse was generated there was only a single founder compared to other injections where there are usually at least 10% transgenic animals in the first litter (Voncken, 2003), and the LAT founder has deleted the SV40 poly A sequence. One potential interpretation of this result is that a portion of the LAT region inserted in the context of genomic DNA may be lethal to the embryo. Further studies are necessary to determine if this is the case. However, the LAT transgenic line that was created can be (and has been) used to study LAT regulation and functions provided that it is stable and expressed.

Initial studies by both hybridization and PCR to determine the number of transgene copies inserted indicate that the LAT transgenic line contains a single copy. The fact that only a single copy was inserted is consistent with the possibility that LAT inserts may not be well-tolerated in mice since it is common to have multiple copies of typical transgenes integrated at a single locus of the mouse genome, in some cases more than 100 copies have been shown to be inserted (Ellis et al., 1997).

Screening for the founder mouse determined that the LAT transgenic mouse was LAT positive and SV40 negative. The probe used to screen for LAT in this case encompassed 897 bases of the transgene and would not evaluate the extent of the deletion. In data presented here it was shown by PCR that the deletion is confined to the 3' most end of the transgene and may be as little as 132 bases. Based on figure 2-5b it appears that the deletion does not include the splice acceptor site since the stable 2kb LAT intron can be detected in transgenic DRG.

The LAT intron exists as a stable lariat structure with a half-life of approximately 24 hours (Thomas et al., 2002). The results obtained from Northern blotting transgenic RNA suggest that the intron is present in its entirety and can be stably expressed in DRG but not in the other transgenic tissues examined. The lack of intron signal in non-neural tissues does not mean that LAT is not being expressed. It is possible that the intron is stable only in ganglia or that splicing of the intron is not as efficient in non-neuronal cells.

CHAPTER 3
EXPRESSION PROFILE OF THE LAT TRANSGENE

Overview

The previous chapter presented data that the LAT transgenic mouse was expressing the LAT transgene through the detection of the stable intron in dorsal root ganglion cells by Northern blot analysis. A quantitative expression profile encompassing both neural and non-neural tissues is presented in this chapter. Since the HSV LAT is expressed during latency, we expected that LAT expression is being regulated largely by cellular functions, and we therefore expected to see LAT expression in the transgenic mouse. What was less clear was whether some viral function contributed to the regulation of LAT expression, and since no other viral genes were present in the transgenic mouse to regulate LAT expression we expected the transgenic mouse would be a valuable tool to look at the cellular control of the LAT promoter. During the course of infection, accumulation of LAT intron typically occurs in neuronal cells (Rodahl and Stevens, 1992), suggesting that neurons contain some factor not present in other cells to allow for the expression of LAT or to prevent its repression. If this was the case, then LAT expression should be seen either exclusively in neural tissues or at higher levels in neural tissues of the transgenic mouse. Therefore, examining LAT expression in neural vs. non-neural cell types was a high priority goal for this investigation.

The two previously described HSV transgenic mice have used non-quantitative methods to examine expression of the LAT transgene. Both the HSV-2 LAT transgenic and the HSV-1 LAT intron transgenic used a Northern blot to assess transgene

expression, and only a few different tissues were examined. In the case of the HSV-2 LAT transgenic mouse, detailed expression data was presented only for some central and peripheral nervous tissues and expression in non-neural tissues was mentioned in the discussion, but data was not presented (Wang et al., 2001; Mador et al., 2003).

In addition to determining the tissue-specific expression profile, the cell-specific expression of LAT was examined by *in situ* hybridization. The Margolis group reported that during an experimental HSV-1 infection of mice, the LAT is expressed in a particular subset of neuronal cells of DRG and TG (Yang et al., 2000). By *in situ* hybridization analysis of the LAT transgenic mouse, we sought to determine if LAT is being expressed in all cells or a subset of cells as evidence of whether LAT expression in different neurons is controlled primarily by cell-specific factors or whether trans-acting viral factors may contribute to the differential expression profile noted by Margolis.

Materials and Methods

Harvesting of Transgenic Tissues

Transgenic mice were euthanized with halothane and cortex, hypothalamus (cerebrum bottom), cerebellum, spinal cord, olfactory bulb, TG, DRG, spleen, liver, kidney, skin, foot, heart, intestine, eye, and lung were dissected, snap-frozen in liquid nitrogen, and stored at -80°C until processed for RNA.

Isolation of RNA from Tissues

Tissues were homogenized in 400 µL Trizol reagent (Invitrogen) using Kontes glass tissue grinders (Fisher) or a ceramic mortar and pestle for larger tissues (i.e. skin, feet, liver). Sterile sand was added to the mortar to aid in grinding the feet and skin. Grinders were rinsed twice with 400 µL Trizol each and the rinse solution was added to

the ground tissue fraction. After a 5 minute room temperature incubation, 240 μL of chloroform was added and homogenates were vortexed 15 seconds followed by a 5 minute incubation at room temperature. Tissue homogenates were centrifuged at 9,000 x *g* for 15 minutes at 4°C, and the aqueous phase containing the RNA removed to a separate tube. The bottom Trizol layer was stored at -80°C for back extraction of DNA if necessary.

RNA was precipitated by the addition of 500 μL of isopropanol and incubation at room temperature for 10 minutes followed by centrifugation at 12,000 x *g* for 10 minutes at 4°C. RNA pellets were washed with 1 mL 70% ethanol and centrifuged 5 minutes at 4°C, 7,500 x *g* and the resulting pellet air dried briefly and resuspended in 200 μL diethyl pyrocarbonate (DEPC, Sigma) treated water.

DNA contamination of the RNA was reduced using DNA-free (Ambion, Austin, TX). One half of the tissue RNA was added to 2 units of DNaseI and 0.1 volumes of DNaseI buffer, mixed gently and incubated at 37°C for 30 minutes. After the incubation, 0.1 volumes of DNase-inactivation reagent was added to each tube and incubated at room temperature for 2 minutes. DNase inactivation reagent was pelleted at 1,000 x *g* for 1 minute and supernatant was transferred to a new tube. RNA was then quantitated spectrophometrically.

Reverse Transcription of Tissue RNA

cDNA was prepared from tissue RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLvRT) and random hexamer priming. For each tissue sample, 500 ng total RNA was added to 4 μL 5x RT buffer (Invitrogen), 10 pmol random hexamers, 12.5 μM each dATP, dTTP, dGTP, dCTP, 200 units MMLvRT (Invitrogen)

and 20 units RNasin (Promega) in a final volume of 20 μL. Reactions were incubated at 37°C for 1 hour followed by 10 minutes at 100°C to inactivate the reverse transcriptase and then ice quenched.

Real Time PCR Reactions

The amount of LAT RNA in each transgenic mouse tissue sample was quantitated using real time PCR and compared to cellular control RNA as described below.

Primer and probe sequences

Primer and probe sequences for the transgene were 5'LAT forward: 5'GGC TCC ATC GCC TTT CCT, 5'LAT reverse: AAG GGA GGG AGG AGG GTA CTG, 5' LAT probe: 5'TCT CGC TTC TCC CC. The location of these primers was diagramed in figure 2-3.

18s RNA was used as a cellular control. The primer and probe set was obtained from Applied Biosystems (PN 4308329). Control 18s RNA was provided with the 18s kit and was reverse transcribed in the same manner as the tissue RNA as described above.

Standards

A standard curve was generated for each of the primer sets used. The pLAT/LAT transgene plasmid was used as standard to determine copy number by performing PCR on 10-fold dilutions of this target ranging from 10^4 to 10^1 copies. The cellular control standard was 18s RNA and was used in 10-fold dilutions 10 ng to 1 pg corresponding to the amount of RNA added to the reverse transcription reaction. This amount was converted to copy number for final analysis.

Conversion of the 18s data from was required because the program associated with the Real Time thermalcycler will not accept numbers for standards that are higher than 10^6. To calculate copy number for 1 ng of 18s the size of mouse 18s RNA (1869 bp)

was obtained from the Qiagen catalog appendix (Qiagen, 2003) and the following formula was used with 6.6×10^{-4} equaling the mass of one base pair.

$$\frac{1\text{ng}}{\mu\text{L}} = \text{fmol} \times 6.6\text{x}10^{-4} \times 1869\text{bp}$$

$$\frac{1\text{ng}}{\mu\text{L}} = 1.23354 \text{ fmol}$$

$$\frac{0.81 \text{ fmol}}{\mu\text{L}} \times \frac{1\text{x}10^{-15} \text{ moles}}{\text{f mol}} = \frac{8.13\text{x}10^{-16} \text{ moles}}{\mu\text{L}} \times \frac{6.022\text{x}10^{23} \text{ molecules}}{\text{mole}} =$$

$$\frac{4.9\text{x}10^{8} \text{ molecules}}{\mu\text{L}}$$

The molecules/ μL were multiplied by the number of copies generated by the real time program to convert ng to molecules.

PCR reactions

All reactions were performed on an ABI Prism 7700 thermal cycler (Applied Biosystems) located in the ICBR protein core at the University of Florida. Samples were run in triplicate. For each reaction, 2 μL of the reverse transcription reaction was added to 0.33 μL 60x Assay Mix (primer/probe set), and 10 μL Taqman Universal PCR Mix (Applied Biosystems part #430437) in a final volume of 20 μL. PCR was performed in 96 well plates under the following conditions: 1 cycle 2 minutes 50°C, 1 cycle 10 minutes 95°C, 40 cycles 15 seconds 95°C, 1 minute 60°C.

In-situ hybridization of Transgenic Tissues

Preparation of tissue sections

DRG, kidney, brain, and spinal cord tissues were harvested from transgenic, non-transgenic littermates, and infected mice and fixed with 4% paraformaldehyde in

phosphate buffered saline (PBS) overnight at 4°C then transferred to 70% ethanol. Tissues were embedded in paraffin blocks and thin sections cut by the pathology core laboratory at the University of Florida. To remove paraffin from cut sections, slides were treated three times for 2 minutes each in Xylenes and then washed twice in 100% ethanol followed by 95%, 70% and 50% ethanol, each for 2 minutes.

Prior to hybridization, slides were treated to remove excess protein and the cellular DNA in the following manner. Fixed tissue was denatured in 0.2M HCl at room temperature for 20 minutes, followed by 2 rinses in distilled water 5 minutes each at room temperature, incubated at 70°C for 30 minutes in 2x SSC, followed by 2 rinses in water 5 minutes each at room temperature. The slides were then treated with Proteinase K (1 μg in 0.02 M Tris pH 7.4, 0.002 M $CaCl_2$) at 37°C for 15 minutes followed by 2 rinses in water 5 minutes each.

DNase pretreatment was performed under treated coverslips. Briefly, coverslips were acid washed in 1 M HCl for 20 minutes then rinsed 3 times in water for 5 minutes each and 3 times in 95% ethanol for 5 minutes each. A final wash for 5 minutes in 100% ethanol dehydrated the coverslips which were then baked for 5 hours in a drying oven at 220°C. For each site, 30 μL of 12U RNase-free DNase (Stratagene) in 20 mM Tris pH 7.4, 10 mM $MgCl_2$ was added and coverslipped. Slides were then incubated at 37°C for 1 hour in a humid chamber. Tissues were rinsed extensively (5x) in 2x SSC 5 minutes each and post fixed in freshly made 5% paraformaldehyde, 0.3 N NaOH in phosphate buffered saline pH 7 for 2 hours in the dark. Excess fixative was removed by washing 3 times in 2x SSC, 5 minutes each and twice in water 5 minutes each. To reduce non-specific hybridization, samples were acetylated in 0.1 M triethanolamine pH 8 with 0.25% vol/vol

acetic anhydride for 10 minutes with stirring then rinsed twice with water for 5 minutes each. A final denaturing step in 95% deionized formamide with 0.1x SSC incubated for 15 minutes at 70°C followed by a 2.5 minutes rinse in ice cold 0.1x SSC and 2.5 minutes in a water rinse.

Preparation of hybridization probes

Probes were prepared from pATD 17 (nt 118,863 to 119,343) and pATD 19 (nt 119,628 to 119,975) plasmids, as diagrammed in figure 2-3, using a random hexamer labeling kit. pATD 17 was digested with PstI and SphI and pATD19 was digested with EcoRI and HindIII to remove the HSV DNA from the plasmid backbone. Both inserts were purified on an agarose gel and the DNA recovered by freeze fracture. For the labeling reaction, 100 ng of digested plasmid DNA was incubated with random hexamers, S^{35} dCTP, cold dGTP, cold dTTP, cold dATP, and Klenow fragment overnight at room temperature according to the random hexamer labeling kit (Roche) specifications. Labeled probes were purified on a Sephadex G-50 spin column to remove unincorporated nucleotides and quantitated by counting 1 μL of labeled probe on a liquid scintillation counter.

Hybridization

The hybridization solution was prepared as follows: 1.5 x 10^5 cpm/site of the labeled probe was ethanol precipitated with 10 μg of salmon sperm DNA, 1/50 volume 5M NaCl and 2 volumes ethanol and incubated at –80°C for 15 minutes. The probe DNA was pelleted by centrifuging for 30 minutes at 4°C, the ethanol was removed, the pellet dried briefly, and resuspended in 20 μL TE. Immediately before use, the probe was heated to 100°C for 5 minutes, followed by quenching on ice. Probes were diluted to 1.5

x 10^5 cpm/site by adding hybridization solution (50% deionized formamide, 0.3 M NaCl, 10 mM Tris pH 7.4, 2x SSC, 1 mM EDTA pH 8, 1x Denhardt's solution, 100 μg/μL denatured salmon sperm DNA, 250 μg/μL, tRNA, and 5 μg/μL polyA) and heated to 100°C for 2 minutes, followed by quenching on ice. To pre-hybridize the probe, the diluted probes were incubated at 45°C for 1 hour, followed by quenching on ice. For the hybridization, 20 μL of the prehybridized probe solution was added to the tissue sections. Slides were covered with a treated coverslip and sealed with rubber cement. Hybridization was carried out at 45°C for 72 hours.

Washes

Cover slips were removed by peeling off the rubber cement from the slides. The slides were first washed in low stringency wash solution (50% crude formamide, 0.3 M NaCl, 10 mM Tris pH 7.4, 1 mM EDTA pH 8) with stirring for 72 hours with 6 changes of wash solution during that period. The first wash change was after 2 minutes to remove excess hybridization solution. After the first wash, a small piece of nylon membrane (i.e. Hybond-N) was included in the washing jar to collect any unbound probe.

After the low stringency wash, slides were washed for 2 hours in high stringency wash solution (50% formamide, 2x SSC, 10 mM Tris pH 7.4, 1 mM EDTA pH 8) at 40°C. Slides were rinsed twice in 2x SSC for 5 minutes each and dehydrated in ethanol (70%, 70%, 95%) containing 0.3 M ammonium acetate to stabilize the counts 5 minutes for each wash.

Filming

To film the slides, NTB-2 nuclear track emulsion (Kodak) diluted 1:1 with 0.6 M ammonium acetate was liquefied at 45°C in the dark. Each slide was dipped into the

emulsion once and allowed to dry approximately 20 minutes before being stood in a slide drying rack for 3 hours with drierite. After slides were completely dry, they were put into slide boxes containing drierite for up to 2 weeks at 4°C. Each slide had a non-hybridized blank slide between it and the next slide to prevent the ^{35}S on one slide from non-specifically exposing the emulsion on adjacent slides.

Developing

Slides were warmed to room temperature for approximately 1 hour before developing. Fresh D-19 developer (Kodak) was used for each experiment. In the darkroom, slides were incubated for 4 minutes in D-19 followed by 3 washes in water for 30 seconds each. Slides incubated in fixative (Kodak rapid fixer) for 4 minutes followed by 2 washes in water 5 minutes each. Slides were counterstained with freshly made Giemsa (Sigma) for 20 minutes, rinsed extensively in tap water and allowed to dry before coverslipping and sealing with Permount.

Statistical Analysis

All data was analyzed using GraphPad In Stat software version 3.05 for the Macintosh computer.

Results

Determination of the Amount of LAT Expressed in Non-Neural vs. Neural Tissues

Comparison of transgene expression on a per weight basis

The amount of LAT RNA present in transgenic tissues was determined by real time PCR. Real time PCR is more accurate than traditional PCR methods because it allows for data collection at the exponential phase of amplification, as opposed to conventional PCR, which measures only the final amount of PCR product. Since conventional PCR endpoints often represent reactions that may have plateaued many

cycles earlier, quantitative comparisons must be made following a series of dilutions of the DNA targets, to insure that comparisons are being made under conditions where all of the PCR products reflect a linear correspondence with the amount of target present in each sample. Since real-time PCR measures the rate of product formation, linear ranges of comparisons of all samples and standards are easily made. For each tissue, 500 ng of total RNA was reverse transcribed (RT) and then a fraction of the RT reaction used for PCR with a real time primer and probe set located within the 5'exon region of LAT (see Figure 2-3). Quantitation of samples was standardized by generating a standard curve from PCR reactions containing known quantities of pLAT/LAT plasmid DNA.

Figure 3-1a represents the expression data presented on a per gram of tissue basis for a group of 4 eight week old mice. Both neural and non-neural tissues exhibited expression of the LAT transgene ranging from 3.51×10^4 to 3.88×10^6 copies/gram of tissue. The variations between the tissues were compared by ANOVA and were not significantly different ($F=1.079$, $P= 0.4017$). Power analysis indicated that more than 100 mice would be needed to attain a statistically significant difference between the expression levels in the transgenic tissues because there was little difference, relative to the error, between the different tissues.

While on the whole there was no significant difference between all of the transgenic tissues, we wanted to look closer at the tissues typically involved in the HSV-1 infection. For these analyses, paired t-tests compared skin with DRG ($t=1.676$, $P=0.1546$) as well as feet with DRG ($t=1.780$, $P=0.1253$) demonstrated no significant difference between either pair of tissues.

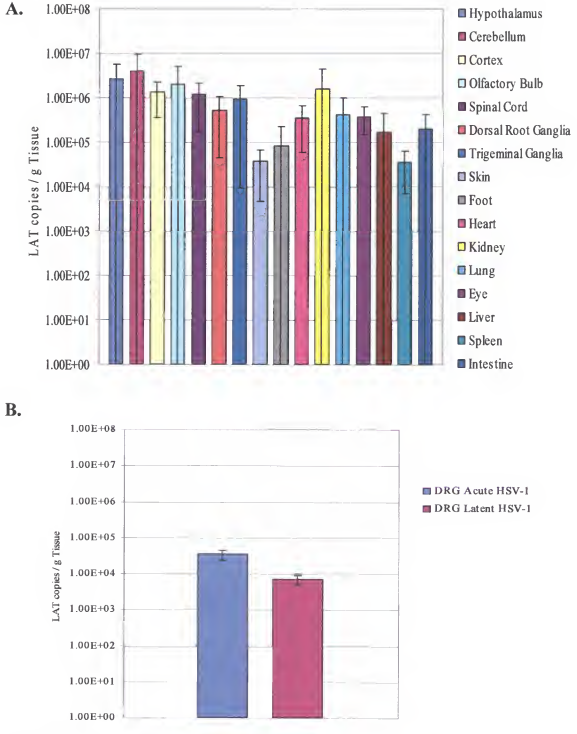

Figure 3-1. Expression of LAT per gram of tissue. A. Both neural and non-neural tissues expressed high levels of the LAT transgene although there was no significant difference between the tissues. For each tissue n=4. B. Expression of LAT during the acute and latent HSV-1 infection.

Comparison of LAT expression in the various tissues normalized to levels of 18s RNA

Although there was no significant difference in LAT expression between the different tissues when compared on a per weight basis, this calculation did not take into consideration that different tissues are composed of different cell types and each cell type has a different density. As a result, comparison by weight, while typically accepted as a basis for comparison did not represent the expression of LAT per cell. To examine the LAT transgene expression on a per cell basis, RT data was normalized to the amount of 18s RNA present in each sample. For these studies, 18s RNA was chosen as the normalizer because it was a cellular housekeeping molecule that remains fairly constant in all tissues (Thellin et al., 1999). This is also a very abundant RNA species, therefore in order to compare the amount of LAT to the amount of 18s RNA present in each tissue sample, the 18s values were divided by 10^8 copies to be in the range of the LAT transgene expression. The expression profile of the same mouse tissues from figure 3-1 was reanalyzed in figure 3-2 on the basis of 18s RNA. ANOVA determined that there was no statistical significance between the transgenic tissues when normalized to the amount of 18s RNA present in each tissue ($F=1.308$, $P=0.2274$).

Comparing the tissues involved in the HSV infection, feet vs. DRG ($t=1.202$, $P=0.2746$) or skin vs. DRG ($t=0.9631$, $P=0.3797$) on a per 18s RNA basis again resulted in no significant difference in the amount of LAT expression between these tissues. The implications of this finding are considered in the Discussion section.

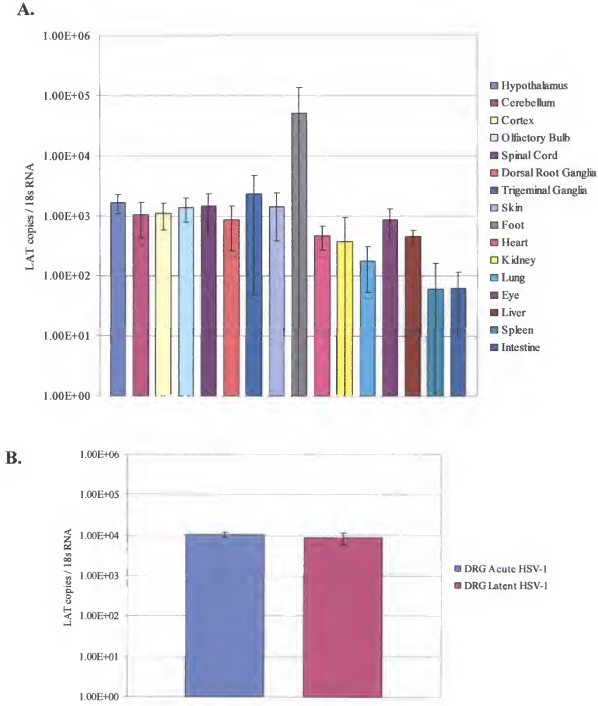

Figure 3-2. Expression of LAT normalized to18s RNA. A. Taking into account the different cell densities in different tissues, LAT expression in transgenic tissues was compared on a per cell basis by dividing the copies of LAT by 10^8 copies of 18s RNA in each tissue. n=4. B. Comparison of the amount of LAT in infected DRG at acute and latent times post infection normalized to copies of 18s RNA.

Comparison of the Amount of LAT Expressed in the LAT Transgenic Mouse to the Amount of LAT Expressed in the DRG During HSV Infection

In the HSV-1 infection not all the DRG cells are infected and of the infected cells only a portion detectably express LAT (Rodahl and Stevens, 1992). To compare the amount of LAT being expressed in the transgenic mouse to the amount of LAT in an infection, we infected mice with 17+ HSV-1 (wild type) and harvested DRG at 4 days post infection (d.p.i.) (acute) and 28 d.p.i. (latent). These ganglia were processed to isolate RNA and LAT expression was quantitated by RT-PCR as described above. Figure 3-1b and 3-2b illustrate the amount of LAT present in DRG during the acute and latent infection compared to the amount of LAT present in the transgenic tissues in Figure 3-1a and 3-2a respectively. The transgenic DRG contained 10-fold more LAT expression per gram of tissue than either the acute or latent infected DRG. When compared on a per weight basis there was no statistical difference between the amount of LAT expressed in the transgenic DRG and either the acutely infected DRG ($P= 0.1448$) or the latently infected DRG ($P= 0.1204$). When we looked at the amount of LAT in the infected tissues on a per cell basis, there was approximately 10 fold more LAT in the infected tissues than in the transgenic DRG. Statistically, the difference between the transgenic DRG and the latent DRG was not quite significant ($P= 0.0786$) while the difference between the transgenic DRG and the acute DRG was significant ($P= 0.0283$) when compared relative to the amount of 18s RNA (Figure 3-2). While we would have expected there to be more LAT in the transgenic tissue compared to the infection, it has been shown that ganglionic levels of LAT are highest at the peak of the acute infection and decline as latency is established. If this is the case, the differences in LAT

expression between the transgenic and infected tissues are minimal. This will be discussed further in the discussion section.

Analysis of Transgene Expression as a Function of Age

Previous studies with HSV-1 transgenic mice expressing reporters behind ICP0 and ICP4 promoters demonstrated a difference in expression of these two lytic gene promoters as a function of the age of the mice (Mitchell, 1995; Loiacono et al., 2002). To determine if the LAT transgenic mouse exhibited an age related expression pattern, we compared the amount of LAT RNA present in selected tissues at 1 day, 1 month, 2 months, and 18 months of age. A representative sample of these data is presented in figures 3-3 and 3-4 while the profile for the entire tissue sampling is located in Appendix C. There was no age related general trend of LAT expression among all of the tissues tested. When compared by weight, the spinal cord ($P= 0.3303$), DRG ($P= 0.6908$), TG ($P= 0.4050$), skin ($P= 0.1728$), and feet ($P= 0.0657$) had no significant difference in the amount of LAT transgene expressed at different ages. This remained the same when comparing LAT expression on a per cell basis using 18s RNA as a reference, with the P-values of the spinal cord ($P= 0.2859$), DRG ($P= 0.4803$), TG ($P= 0.2429$), skin ($P= 0.5305$), and feet ($P= 0.5707$) indicating a lack of significance (figure 3-4).

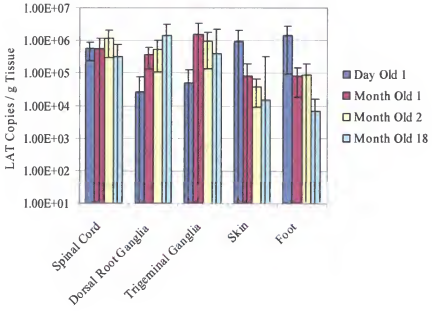

Figure 3-3 LAT transgene expression is not age dependent in tissues typically involved in the HSV infection when normalized on a per weight basis.

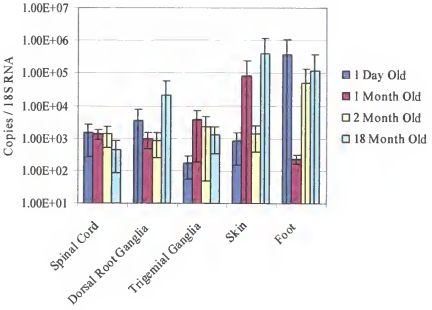

Figure 3-4. Expression of the LAT transgene is not age dependent in tissues involved in the HSV infection when calculated per cell by normalizing to 18s RNA.

Analysis of Transgene Expression in Neural and Non-neural Tissue at the Cellular Level Using *In situ* Hybridization

In situ hybridization examined two properties of expression, the number of cells expressing the transcript of interest and the cellular localization of the transcript (nuclear or cytoplasmic). To determine if the LAT transgene was being expressed in all cells of transgenic tissues we probed for either the 5' LAT exon or the LAT intron by RNA *in situ* hybridization. These tissue sections were counterstained with Giemsa stain after hybridization which is a general membrane stain. In DRG, neurons were visible as large blue round nucleated cells. The tissue between the groups of neurons included support cells and the axons leading to the neuron cell bodies. Figure 3-5 illustrates the architecture of the DRG magnified to point out the location of the nucleus seen as a white

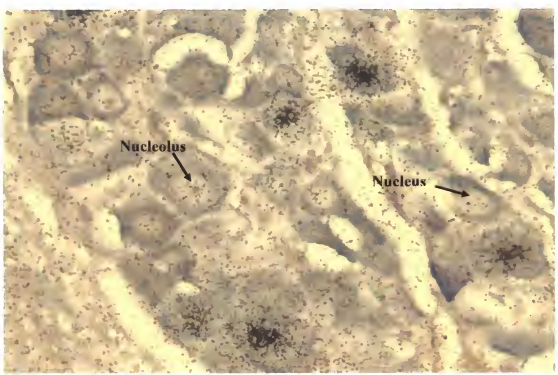

Figure 3-5. *In situ* hybridization of a latently infected dorsal root ganglia. This photograph illustrates the neurons of the dorsal root ganglia (blue) and points out the sub-cellular architecture with arrows pointing to the nucleus and nucleolus of neurons. The small black dots on some of the cells are the positive hybridization signal.

or lighter stained region within the neurons and the nucleolus seen as a gray circle within the nucleus of some neurons.

The DRG is the primary site of LAT expression during the HSV infection following footpad inoculation. Figure 3-6a illustrates the nuclear localization of expression of the LAT 5' exon during the latent infection, marked by black arrows. Surprisingly, in the transgenic DRG there were a subset of neurons that express LAT with a different localization pattern than the infected DRG (compare figure 3-6a black arrows with figure 3-6b red arrow) and some of the expression appears to be cytoplasmic. This sub-population was in addition to the nuclear staining cells (figure 3-6b black arrows) in the transgenic tissues that have the same pattern as the HSV infected DRG. A non-transgenic mouse exhibited only background signal in this experiment (figure 3-6c). When probed for the LAT intron, transgenic DRG showed the nuclear localization of high levels of expression and low levels of expression in neurons as seen in Figure 3-7. The cytoplasmic localization of LAT was not detected with the intron probe.

Neurons were counted to quantitate the positive sub-populations of neurons for both the infected and the transgenic tissues with the 5' exon probe. During the HSV infection LAT has been shown to be expressed in approximately one third of latently infected neurons (Gressens and Martin, 1994; Maggioncalda et al., 1996). The data presented here illustrated that LAT was being expressed in slightly more than two thirds of transgenic neurons compared to just less than one third of neurons in infected dorsal root ganglia (Table 3-1). Additionally, the transgenic tissue expression can be further

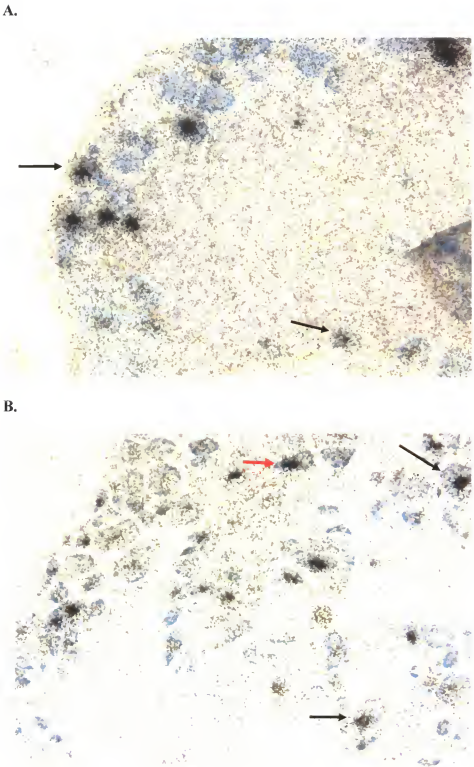

Figure 3-6. *In situ* hybridization for 5' LAT exon. A. Latently infected dorsal root ganglia. B. Transgenic positive dorsal root ganglia. C. Transgenic negative dorsal root ganglia. Black arrows represent hybridization of nuclear LAT

C.

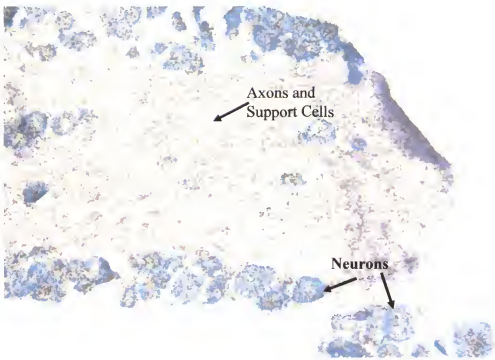

Figure 3-6 (cont'd). expression. Red arrow indicates cytoplasmic LAT expression. Arrows in C point out the neurons and axons of the DRG.

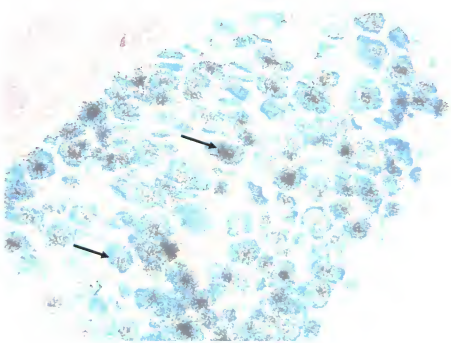

Figure 3-7. *In situ* hybridization for LAT intron in transgenic DRG. Black arrows represent nuclear localization of hybridization at both high and low levels of expression.

divided into three subpopulations, darkly stained or high expression (33%), weakly stained or low expression (56%), and cytoplasmic stained (11%). Further studies are needed to determine if these subpopulations coordinate with neuronal markers for neuronal subpopulations.

Table 3-1. Quantitation of LAT Expressing Cells in Dorsal Root Ganglia by *in situ* hybridization for the 5' LAT exon

	Number Positive	**Percentage Of Total**
Latently Infected[1]	204	25
Transgenic Tissues Totals[2]	709	65
Darkly Stained	233	[3]33
Weakly Stained	399	56
Cytoplasmic Stained	77	11

[1]Total neurons counted = 806 [2]Total neurons counted = 1095 [3]For the subsets of positive neurons in the transgenic, percentage is of total positive transgenic neurons.

A significant finding of this study is that the 5' LAT expression pattern seen in the transgenic DRG was not seen in other tissues of the transgenic mouse. A comparison of other neuronal tissues of the transgenic mouse demonstrated a small amount of detectable LAT expression in the thalamic neurons of the brain (figure 3-8 and figure 3-9) with considerably less intensity than in DRG (compare figure 3-6b with figure 3-8 and 3-9). Additionally, a small number of neurons in the spinal cord (figure 3-10) were expressing LAT to levels comparable to that in the DRG. As with the DRG, further studies are necessary to determine if these hybridization positive cells are from a specific population

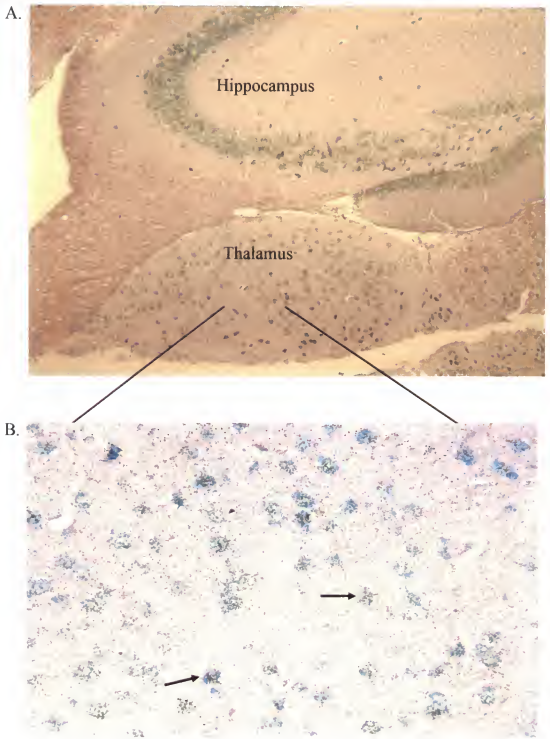

Figure 3-8. *In situ* hybridization for LAT 5'exon in brain. A. Transgenic brain 10x B. Transgenic brain magnifying the thalamic region 40x.

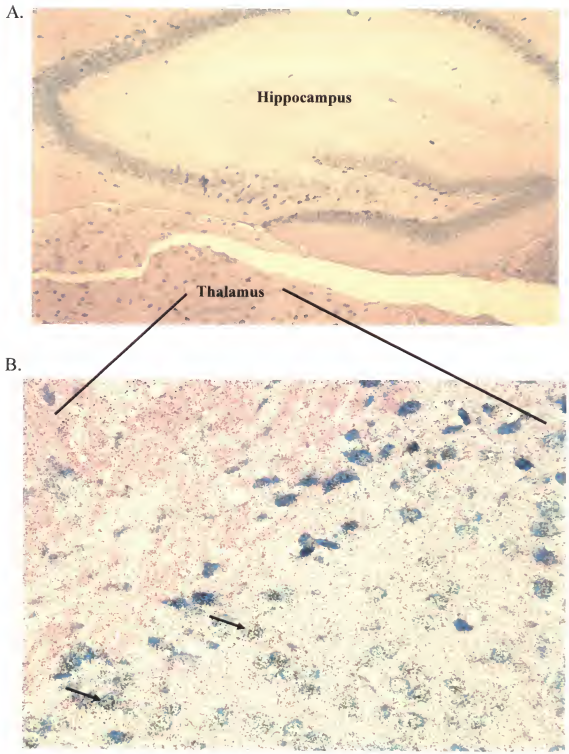

Figure 3-9. *In situ* hybridization of the transgenic brain with LAT intron probe. A. Low magnification for orientation purposes. (10x) B. Positive signal (black arrows) was detected in thalamic neurons with primarily lower levels of expression than seen in the DRG. (40x)

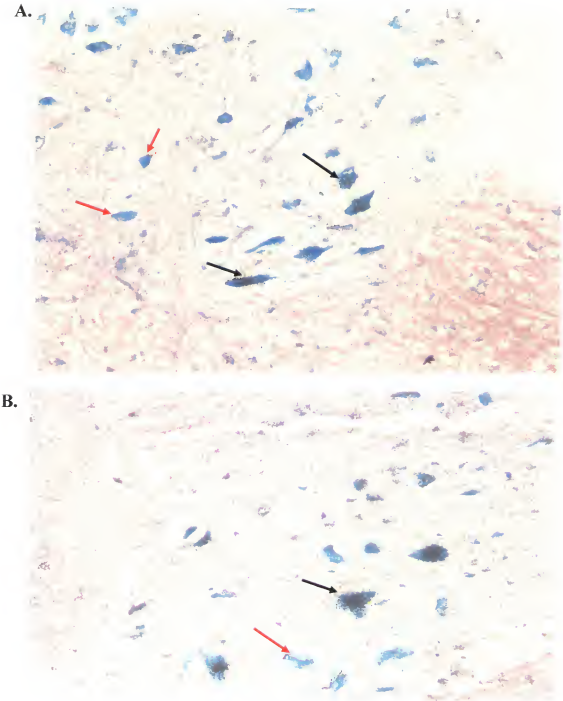

Figure 3-10. *In situ* hybridization in the transgenic spinal cord. A. 5' LAT exon probe. An example of positive neurons is marked with black arrows and examples of negative neurons are marked with red arrows. B. LAT intron probe.

of neurons. In contrast to the QRT-PR data for the non-neural tissues, both the transgenic foot (Figure 3-11) and the transgenic kidney (Figure 3-12) exhibited no detectable LAT expression by *in situ* hybridization. The cell specific distribution of LAT expression exhibited by the transgenic mouse in both neuronal and non-neuronal tissues has important implications for the final analysis of the overall LAT expression levels in the different tissues of the transgenic mouse. This will be discussed in detail in the following section.

Discussion

The LAT was able to be expressed in the absence of other HSV proteins in the transgenic mouse. We had expected to see this expression primarily in neural tissues (brain, spinal cord, ganglia) and possibly some epithelial tissues as well, for example skin and foot, which have demonstrated some LAT expression during the HSV infection (Jarman et al., 1999). The data presented here show that LAT can be detected at relatively high overall levels, in a variety of tissues in the transgenic mouse. The tissues expressing LAT were consistent with the expression patterns seen in the HSV-2 transgenic mouse (Wang et al., 2001) that was under control of the native HSV-2 LAT promoter. We were unable to compare the amount of expression between the LAT HSV-1 and HSV-2 mice because quantitative data were not reported in the HSV-2 mouse.

An important consideration, however, is that the analysis of the expression data on a per weight basis did not take into consideration that different cell types have different densities and cell compositions, although this is the standard method for representing such data. To provide a per cell based analysis, we compared the expression data to 18s RNA levels. Selection of a cellular gene to be used as a control must be done

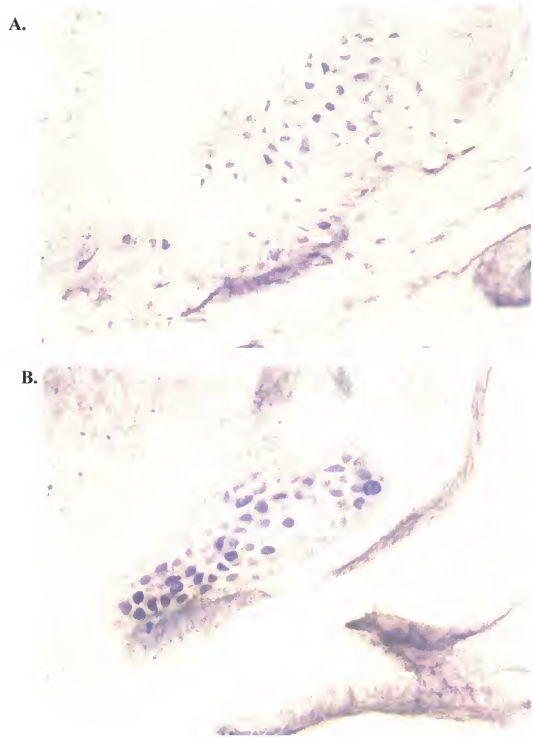

Figure 3-11. *In situ* hybridization of transgenic foot. A. LAT 5' exon probe B. LAT intron probe. There was no hybridization signal with either probe in the LAT transgenic foot.

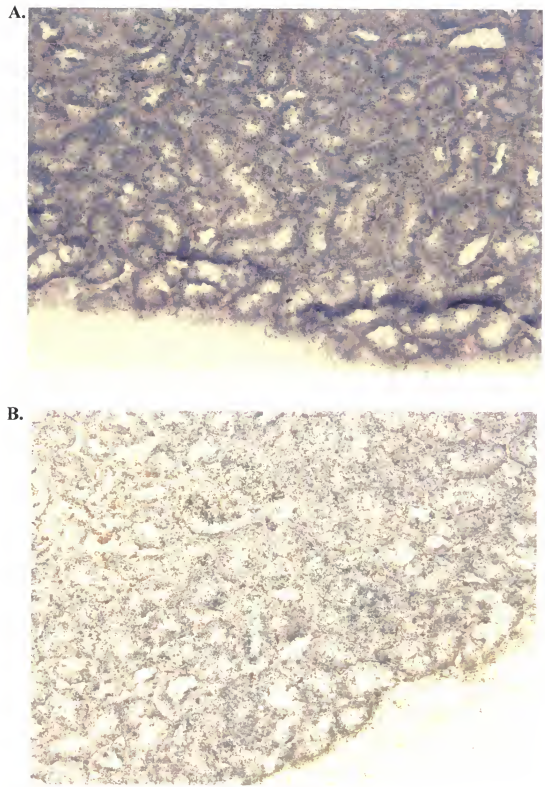

Figure 3-12. *In situ* hybridization in the transgenic kidney. A. LAT 5' exon probe B. LAT intron probe. No positive hybridization signal was detected with either probe.

with care since a number of cellular genes exhibit varied expression in different cell types. In our studies these differences would have had an effect on the quantification of LAT expression in the different tissue types. The 18s RNA species is a cellular housekeeping gene that is considered to be constant in all cells (Thellin et al., 1999) allowing for comparison between tissues.

Similar to the per weight analysis, LAT expression had no significant difference between tissues. Despite of the inability to detect a difference in expression by either analysis, the 18s RNA comparison appeared to be a more accurate overall representation at the cellular level. The per weight analysis of expression would be sufficient for more general comparisons but is not quantitative when expression may be present in only certain cell types of a complex tissue.

There was a statistically significant difference between the amount of LAT in the acute infection and the amount of LAT in the transgenic DRG when compared on a per 18s basis. The comparison with the latent infected DRG was not quite significant but there was more LAT produced in the infected tissue than in the transgenic DRG. In combination with the *in situ* data which suggests that LAT is not being produced in every cell of the DRG, it is not hard to conceive that there could be more LAT in the infected tissue. Further studies are needed to determine if this is the case, however, the 18s RNA comparison remains a valid comparison between the non-infected tissues of the transgenic mouse since they are comparing LAT that is being controlled in the same manner.

We have also shown that LAT was not expressed in an age dependent manner. Studies of immediate early HSV genes in transgenic mice determined that both ICP0 and

ICP4 were expressed with age dependent differences (Mitchell, 1995; Loiacono et al., 2002). There were a number of factors that made the comparison of the LAT and the immediate early studies difficult. In the immediate early study they were counting the number of cells expressing a β−gal reporter, not measuring the amount of β−gal expressed. Our 18s RNA analysis would suggest that there was not a great variation in the number of cells expressing the LAT transgene, but again, the two quantitations may not be directly comparable.

Interestingly, LAT is not expressed in all cells of the transgenic mouse. *In situ* hybridization for the LAT transgene illustrated that in the DRG, the transgene was being expressed in only a subset of neurons. The pattern of LAT expression in the DRG of transgenic mice seems to differ from the expression in the HSV-1 infection. In the transgenic DRG we can detect two intensities of LAT expression, both nuclear in localization, that were similar to the infected DRG, although more abundant. Additionally, there was a small fraction of cytoplasmic localization of LAT expression in the transgenic mice when using the LAT 5' exon probe but not with the LAT intron probe. It is not clear whether these observed differences are related to LAT expression differences by the transgenic mouse, or due to influences of other viral factors that may modulate LAT expression in these neurons during a normal HSV infection. In addition, further studies are needed to determine if these neurons have a particular characteristic or if expression is in a random subset of neurons. Based on the expression patterns of LAT in infected DRG and on the *in situ* data from the spinal cord in seems more likely that the expression positive neurons in the transgenic mouse should have a particular characteristic.

In other transgenic tissues, LAT expression was not detectable by *in situ* hybridization with either the 5' LAT exon or LAT intron probes. The kidney and foot both had transgene expression when measured by RT-PCR for the 5' exon but expression was not seen by *in situ* hybridization. This could suggest that in some tissues LAT expression is leaky in all cells and thus below the level of sensitivity for the *in situ* but, in the neuronal tissues expression is confined to a subset of neurons resulting in the ability to detect the expression by *in situ* hybridization. The small number of cells expressing the LAT transgene in the spinal cord appears to support this theory in addition to the expression in the DRG. These findings may suggest that LAT expression is more tightly and dramatically regulated in sensory neurons.

CHAPTER 4
CHARACTERIZATION OF THE EXPRESSION OF LAT *IN TRANS* ON THE COURSE OF HSV-1 INFECTION IN MICE

Overview

HSV-1 infection is characterized by three phases; acute, latent and reactivation. The LAT has been suggested to play some role in all three phases, although its most striking phenotype seems to be primarily at the level of reactivation (Wagner, 1991; Bloom et al., 1994; Bloom et al., 1996b; Wang et al., 1997). The mechanism of its action in this process has not been determined. Limited amounts of LAT have also been seen during the acute phase of infection, although at present, there is no function linked to this expression (Jarman et al., 1999).

If the LAT RNA used a *trans* mechanism of action, exerting a function on an HSV-1 DNA molecule that had not actually produced the LAT RNA, then we would expect to see a phenotype at some phase of the HSV infection in the LAT transgenic mouse. For example, we could experimentally infect the LAT transgenic mouse with a LAT(-) virus and phenotypically convert the virus to resemble wild type. Contrarily, if LAT functions in *cis*, functionally regulating or interacting with the DNA molecule that produced the LAT RNA, then there would be no visible phenotype when infecting the LAT transgenic mouse with HSV-1. With this in mind, experiments that examined the acute, establishment and reactivation phases of the HSV-1 infection were performed in the LAT transgenic mouse. For the acute studies, LAT transgenic mice and their non-transgenic littermates (as controls) were infected with HSV-1 on their rear footpads. The

relative progression of the acute infection was assessed by the yield of infectious virus in the feet, spinal ganglia, and spinal cord at selected time points post infection. Establishment of HSV latency was assessed by the amount of HSV-1 DNA present in the DRG of transgenic and non-transgenic mice after the acute infection had resolved. Explant co-cultivation was used as a reactivation model to determine if the LAT transgenic mouse exhibited detectable differences in the ability to reactivate latent HSV.

Finally, deletion of a portion of the LAT region has been shown to dramatically reduce the virulence and yield of virus within sensory neurons (Gary et al., in preparation). While it has been shown that this virulence function acts independently of the LAT promoter (which is primarily involved in reactivation), this new function still resides within the region of the LAT gene present in the transgenic mouse. Here we sought to determine if this phenotype could be rescued by using the LAT transgenic mouse to provide the deleted region *in trans* during an experimental HSV infection. Given the dramatic reduction in virulence associated with this LAT deletion, we felt this would be a sensitive test of the LAT's ability to act *in trans*.

Materials and Methods

Growth of Cell lines and Viruses

Rabbit skin (RS) cells were maintained in minimum essential media (MEM) with Earle's salts supplemented with 5% calf serum and antibiotics at 37°C in a 5% CO_2 incubator. HSV-1 strain 17^+ and the HSV-1 mutant 17Δ480 were grown and titered on RS cells. The mutation in 17Δ480 deletes a portion of the LAT intron corresponding to nucleotides 119,502 to 119,981. Characterization of this virus has been described elsewhere (Jarman et al., 2000).

Footpad Infection of Mice

All infections used adult mice of at least 6 weeks of age, HSV-1 LAT transgenics and their transgenic negative littermates of at least the F6 generation. Mice were transferred to the UF animal care infectious disease suite after genotyping, and were allowed to acclimate to their new housing conditions for at least one week prior to infection.

Mice were anesthetized with halothane and subcutaneously injected with 0.1 mL of 10% saline in each of the rear footpads. Four hours post saline pre-treatment, mice were anesthetized with 0.010 to 0.020 mL of a ketamine cocktail (2.5-3.75 mg/kg acepromazine, 7.5-11.5 mg/kg xylazine, 30-45 mg/kg ketamine) intramuscularly in the thigh. Both rear footpads were abraded with an emery board to remove the keratinized layer of skin tissue. Using a pipette tip, 1 x 10^6 plaque forming units (pfu) of virus in 50 μL volume was added to the footpads and allowed to absorb for one hour with mice lying on their backs under anesthesia. Mice were monitored twice daily for signs of complications due to anesthesia or infection.

Harvesting of Infected Tissues

At specified times post infection, mice were euthanized with halothane and infected tissues were dissected (feet, DRG, spinal cord). Tissues were snap-frozen in liquid nitrogen and stored at -80°C until processed.

Determination of Viral Titers from Infected Tissues

Infected tissues were homogenized in Kontes glass tissue grinders (Fisher) or a ceramic mortar and pestle (feet). DRG were ground in 1 mL MEM with supplements and grinders rinsed with 0.4 mL MEM. Spinal cords were ground in 2.5 mL MEM and rinsed with 2.3 mL MEM. Feet were ground with 2.5 mL MEM containing 2x antibiotics and

250 ng/mL fungizone and sterile sand then rinsed with 5 mL MEM. Homogenates were centrifuged at 3000 x *g* for 15 minutes to pellet cellular debris.

Titration dishes were prepared with rabbit skin cells in 24 well plates to be sub-confluent at the time of inoculation. Serial dilutions from 10^{-1} to 10^{-8} of the tissue homogenate supernatants were made in MEM with supplements. Media was removed from the titration plates and 200 µL of each dilution of tissue homogenate was added in triplicate, and virus allowed to absorb for 1 hour at 37°C with 5% CO_2. After the inoculation, plates were rinsed with 2 mL of MEM to remove the inoculum and any residual cell debris and 2 mL MEM was added to each well. Cells were incubated for 72 hours at 37°C with 5% CO_2 then the media was removed and the cells were stained with crystal violet to view plaques. Excess crystal violet was rinsed off with tap water and plates were allowed to air dry before counting.

Determination of the Amount of HSV DNA in Latently Infected Mice

Adult transgenic mice and their non-transgenic littermates were infected as described above with 1 x 10^6 pfu of HSV-1 17^+ (wild type) and monitored for complications. After 28 days, mice were euthanized, DRG removed, and snap-frozen in LN_2 until processing. DRG were ground in 400 µL Trizol reagent in a Kontes glass tissue grinder (Fisher) to extract DNA. Grinders were rinsed twice with 400 µL Trizol each and rinse solution was added to the ground tissue. After a 5 minute room temperature incubation, 240 µL of chloroform was added and the homogenates were vortexed for 15 seconds followed by a 5 minute incubation at room temperature. Tissue homogenates were centrifuged at 9,000 x *g* for 15 minutes at 4°C. At this point, the clear aqueous phase containing the RNA was removed to a separate tube.

RNA was precipitated with 500 μL of isopropanol at room temperature for 10 minutes followed by centrifugation at 12,000 x *g* for 10 minutes at 4°C. RNA pellets were washed with 1 mL 70% ethanol and centrifuged 5 minutes at 4°C, 7,500 x *g*. The resulting pellet was air dried briefly and resuspended in 200 μL diethyl pyrocarbonate (DEPC, Sigma) treated water. RNA fractions were stored at -80°C for later use.

The lower phase (Trizol) of the extractions from the initial centrifugation step was removed from the bottom keeping the interface intact. DNA was back extracted from the remaining interface by adding 150 μL of 0.1M Tris and 0.1% Sarkosyl and centrifuging 5 minutes at 20,000 x *g*. This back-extraction was repeated twice, pooling the aqueous layer after each centrifugation. Proteinase K (0.1 μg/μl) was added to the pooled back-extracted DNA samples and incubated at 37°C overnight. DNA was purified by sequential extractions with an equal volume of phenol and sevag followed by extraction with sevag and precipitation in 100% ethanol. Pellets were resuspended in 50 μL TE and quantitated spectrophotometrically (A_{260}).

The amount of HSV genomes present in each mouse was quantitated by real time PCR using primers and a probe specific for the HSV polymerase (pol) gene. Details of the real time procedure were presented in chapter 3. The HSV pol primer/probe set was custom made by Applied Biosystems, Assays by Design with the forward primer sequence 5'AGAGGGACATCCAGGACTTTGT, reverse primer sequence 5'CAGGCGCTTGTTGGTGTAC and probe sequence 5'ACCGCCGAACTGAGCA (65,880 to 65,953 nt). For the PCR reaction, conditions were 1 cycle 2 minutes 50°C, 1 cycle 10 minutes 95°C, 40 cycles 15 seconds 95°C 1 minute 60°C as described in chapter 3.

A standard curve was generated using HSV-1 genomic DNA of known copy number ranging from 10^5 to 10^2 copies. Unknown samples were assayed using 50 ng of DNA per reaction and were compared to the standard curve values for quantitation.

Explant Co-cultivation of Latently Infected DRG

Latently-infected transgenic and non-transgenic mice were euthanized and DRG dissected as described above. DRG were cultured in MEM on with a RS cell monolayer at 37°C with 5% CO_2 to detect reactivating virus. Every 48 hours, half of the media was removed and replaced with fresh MEM to ensure the integrity of the monolayer. Cells were monitored daily for 14 days for the presence of rounded, HSV infected RS cells.

Results

Expression of LAT in *trans* Does Not Detectably Alter the Course of an Acute HSV-1 Infection in Mice

While the mouse footpad model of HSV-1 infection does not use the natural site of HSV infection, it mimics the natural infection in that HSV can infect the epithelial surface of the foot and travel along the sciatic nerve to reach the DRG. The reason that the footpad model is extensively used to assess virulence properties of HSV-1 strains and to study the progression of the acute infection is that it provides HSV-1 with a longer path to travel through the nervous system until it reaches the brain. Therefore one can sensitively assay the relative replication potential of different strains of HSV as it travels from the foot, to the DRG, to the spinal cord and then to the brain.

To determine if expressing the LAT in the context of the transgenic mouse would affect the course of the HSV-1 infection, we infected transgenic and non-transgenic mice using the footpad model with wild-type HSV-1 strain 17+. The rationale for this experiment is that if LAT is hypothesized to play a role in regulating HSV-1 gene

expression, expression of LAT *prior* to the HSV-1 infection might affect the outcome of the acute infection, if could function in *trans*. Following footpad infection, tissues along the path of HSV infection were assayed for amounts of infectious virus present at acute times post infection, days one to four (figure 4-1). There was no significant difference between the transgenic and non-transgenic mice at any of the four time points tested, day 1 P= 0.3501, day 2 P= 0.2403, day 3 P= 0.5476, and day 4 P= 0.5519. Similarly, the amount of infectious virus in the DRG at acute times post infection shown in figure 4-2, exhibited no significant difference between transgenic and non-transgenic mice, day 2 P=0.7364, day 3 P= 0.4309, and day 4 P= 0.3735. These data suggest that LAT was not

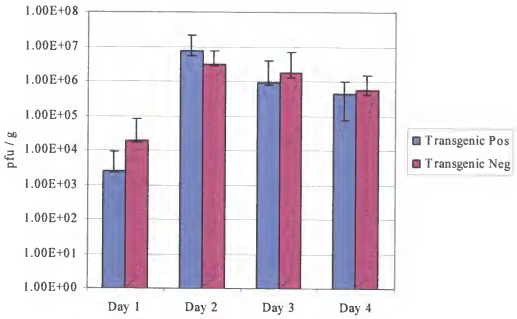

Figure 4-1. Relative amounts of infectious virus in the feet of transgenic and non-transgenic mice infected with HSV-1 strain 17+ during acute times post infection. n= 4

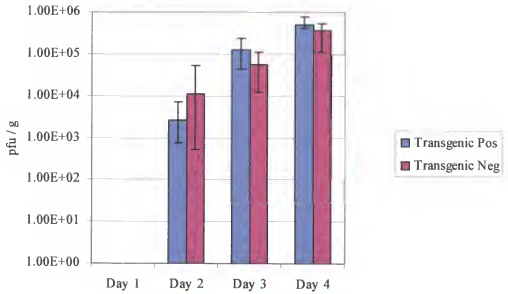

Figure 4-2. Titer of infectious virus in DRG of transgenic and non-transgenic mice infected with HSV-1 strain 17+ at acute times post infection. n= 4

affecting the acute phase of the HSV infection by being expressed at earlier times (*prior* to infection) in the transgenic animals, and the LAT was not acting in *trans* to detectably alter the outcome of the acute phase of HSV-1 infection.

Establishment of HSV-1 Latency in Transgenic Mice

Once the virus reaches the ganglia, it is able to establish latency in ganglionic neurons. Since the LAT has been proposed by some to play a role in the establishment of latency (Speck and Simmons, 1991; Bloom et al., 1994; Bloom et al., 1996a; Wang et al., 1997), the expression of the LAT in the transgenic mice may affect the amount of establishment in these mice. In order to determine if the expression of the LAT transgene altered the amount of HSV that established latency in the infected mice, we infected transgenic and non-transgenic mice with 1 x 10^6 pfu HSV-1 17^+ (wild type) and waited 28 days for latency to be established. Mice were euthanized and DRG harvested and snap frozen in LN_2 until processed to extract the DNA. HSV DNA was quantitated

using real time PCR primers for the HSV polymerase. In figure 4-3 the amount of HSV DNA present in ganglia of transgenic and non-transgenic infected mice, illustrated no difference between the two groups (t= 0.1623, P= 0.8722). The mean for each group is represented by a horizontal line. Scatter within the groups was expected because there is some variability in the amount of virus infecting each mouse. Despite the expression of the LAT from the transgenic mouse, there was no detectable difference in the amount of establishment of HSV latency between the transgenic and non-transgenic mice, thus expression of the LAT in *trans* did not affect the amount of HSV reaching the DRG and establishing a latent infection.

Figure 4-3. Quantitation of HSV-1 genomes present in mice infected with HSV-1 17+ at latent times post infection. Each symbol represents one mouse.

Reactivation from Latency in Transgenic Mice

The LAT also plays a role in reactivation from latency (Wagner, 1991; Bloom et al., 1996b; Hill et al., 1996; Jarman et al., 2002). During reactivation, the virus initiates a

productive cascade resulting in the generation of progeny virus. One of the methods for measuring the presence of reactivatable virus in the mouse model is explant co-cultivation. In this process, DRG were removed from latently infected mice and co-cultured in tissue culture media until progeny virus was released and detected by the presence of CPE on the RS monolayer. In the human or rabbit eye infection, reactivated virus would travel along the axons to the initial site of infection. When using the mouse explant model, those axons have been dissected away and the virus travels into the media from the tissue. This virus can then infect tissue culture cells that are in the dish with the ganglia to serve as a detection method for reactivation.

To study the effect of the LAT transgene on the reactivation phase of infection, DRG from latently infected mice were dissected and cultured in tissue culture media on a layer of rabbit skin cells for 14 days. Cultures were monitored daily for the presence of infected rabbit skin cells. In both the transgenic and non-transgenic mice, virus was detected in all of the cultures by day 10 post co-cultivation (figure 4-4). There was also no observed difference in the time frame of reactivation between transgenic and non-transgenic mice.

Expression of the Transgene in *trans* Does Not Rescue the Restriction of a LAT Deletion Mutant in Neural Tissue

A region of the LAT transcript has been shown to play a role in the virulence of HSV-1. When this region of the virus was deleted, the resulting virus was markedly decreased in virulence (Gary et al., in preparation). The LAT transgenic mouse includes the region that was deleted in this virus, 17Δ480, and could provide that function during the course

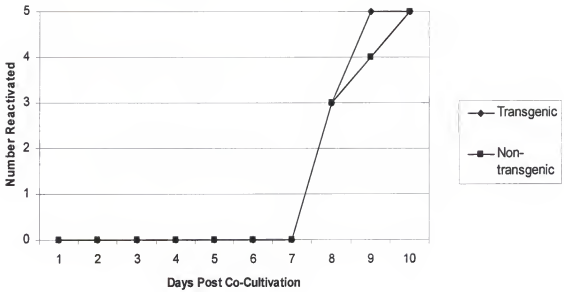

Figure 4-4. Reactivation of HSV-1 from transgenic and non-transgenic mice by explant co-cultivation of latently infected DRG.

of infection in the mouse. The location of this deletion in relation to the mapped virulence function of LAT and the LAT transgene is diagramed in figure 4-5. To see if providing the deleted LAT region in *trans* would rescue the virulence phenotype, we infected transgenic mice with the 17Δ480 virus in the rear footpad. At acute times post infection, days one to four, feet and DRG were harvested, homogenized and titered for infectious virus. Both transgenic and non-transgenic mice exhibited similar levels of virus in the feet, seen in figure 4-6, with no significant difference between the groups at any timepoint. Day 1 P= 0.1990, day 2 P= 0.3281, day 3 P= 0.9528, day 4 P= 0.5585.

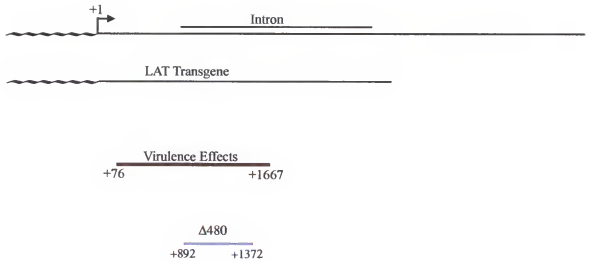

Figure 4-5. Diagram of the LAT region of HSV illustrating the location of the 17Δ480 virus and the LAT transgene in relation to the virulence function of LAT.

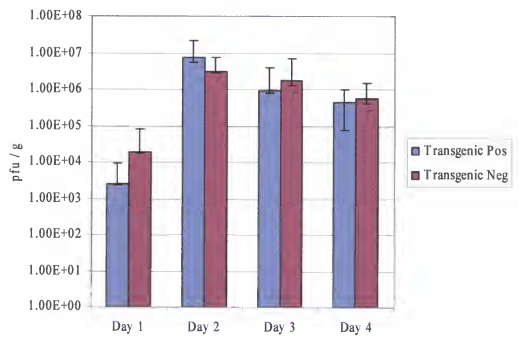

Figure 4-6. Relative amounts of infectious virus in feet of transgenic and non-transgenic mice infected with a deletion mutant of HSV-1 at acute times post infection. n=4

In the DRG, figure 4-7, the increase seen with the transgenic mice was not significant, day 2 P= 0.3029, day 3 P= 0.3964, day 4 P= 0.0727. Since only four mice were tested per group in these experiments it is possible that a larger sample size would have resulted in a significant rescue of the virulence phenotype. The initial virulence studies used Swiss Webster mice, which are less resistant to HSV infection. Thus, to prevent genetic differences from complicating the results, the transgenic mouse is being bred into the Swiss Webster background prior to repeating these experiments.

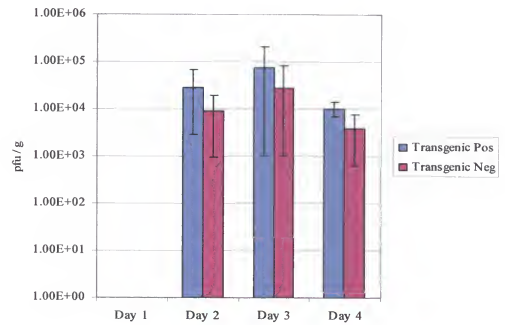

Figure 4-7. Relative amounts of infectious virus in DRG of transgenic and non-transgenic mice infected with a deletion mutant of HSV-1 at acute times post infection. n= 4.

Discussion

The LAT has been proposed to play a role in the establishment and reactivation phases of the HSV infection. The LAT transgenic mouse expressed LAT to high levels in a number of tissues, as presented in chapter 3. We hypothesized that the expression of LAT by the transgenic mouse in a temporally different manner from what occurs during

the normal HSV infection could have an effect on either the establishment or reactivation of latency. We examined all three phases of the HSV infection in the transgenic mouse. During the acute infection there was no difference in the amount of infectious virus present in the feet or DRG of transgenic and non-transgenic mice. The amount of HSV DNA present in latently infected DRG of the transgenic and non-transgenic mice was comparable, indicating that the LAT transgene was not detectably affecting the establishment of a latent infection, at least by the criterion of the presence of HSV-1 genomes. Co-cultivation measured the ability of these genomes for biological activity and their ability to reactivate and, again no difference between transgenic and non-transgenic mice was observed. Thus, expression of the LAT transgene prior to infection in the mouse had no detectable effect on any phase of the HSV infection.

The LAT region also encodes a virulence function that seems to be genetically distinct from LAT's reactivation function, as seen with deletion mutants in the region of the intron illustrated in figure 4-5. The transgenic mouse contains the region of HSV DNA corresponding to this virulence function. When infecting transgenic mice with the deletion mutant, 17Δ480, there was no significant restoration of the wild type virulence levels during the acute infection, although some slight differences were observed. Since the transgenic mouse is in the more resistant C57Bl/6 background, there could be other genetic factors involved in the virulence function that was initially discovered by infecting more sensitive Swiss mice. The data presented suggest that there may be a slight restoration of virulence, although the difference was not statistically significant, in the transgenic mouse which will be further studied in the Swiss background after backcrossing of the transgenic mouse with Swiss mice to obtain a Swiss genetic

background transgenic line. It should be pointed out, however, that the dramatic restriction in ganglionic replication exhibited by 17Δ480, and the fact that this restriction is also detected on PC-12 cells in culture would seem to suggest that it is unlikely that the lack of rescue was due to a difference in mouse background.

Taken together, these data suggest that LAT does not act in *trans* to regulate its functions. A more likely model is that LAT is acting in *cis* to regulate other functions in the HSV-1 genome. Given the proximate location of the LAT to ICP0 and ICP4, these might be candidates for this *cis*-acting activity.

CHAPTER 5
OVERALL CONCLUSIONS

The LAT is the only transcript detected abundantly during the latent phase of HSV-1 infection (Roizman and Sears, 1996). This transcript has been implicated as a modulator of a number of viral functions including, but not limited to, establishment of latency (Speck and Simmons, 1991), reactivation (Wagner, 1991; Bloom et al., 1994; Bloom et al., 1996), virulence (Perng et al., 1999), and neuronal survival (Perng et al., 2000; Thompson and Sawtell, 2001; Ahmed et al., 2002). One of the key features of the LAT is that it exhibits a differential tropism of expression with only 1/3 of latently infected neurons expressing large amounts (Maggioncalda et al., 1996). In addition, LAT-expressing neurons seem to segregate into subsets of sensory neurons expressing specific markers. Since the degree to which the regulation of LAT expression is dictated by phenotypically different classes of neurons is not well understood, we sought to study the regulation of LAT expression in the absence of other viral proteins by generating a transgenic mouse that contains the LAT region.

Characterization of the transgenic mouse described in this dissertation has shown that the LAT transgene exists in a single copy in the mouse genome and that it is expressed to high levels in a number of tissues. This overall expression, as assessed by total LAT RNA detected at the level of whole tissues, does not seem to be neuronal-specific since expression was seen in non-neural tissues as well. This seemed somewhat surprising to us initially since the literature contains numerous studies showing that LAT has higher levels of expression in neuronal cells than in other cell types (Zwaagstra et al.,

1990; Kenny et al., 1994; Dobson et al., 1995; Coffin and Thomas, 1998; Jarman et al., 1999). *In situ* hybridization shed some light on this apparent incongruity by demonstrating that the transgene was being expressed strongly in only a subset of neurons in the dorsal root ganglia. Expression was not detected by *in situ* hybridization in non-neural tissues. Thus, it can be concluded that strong LAT expression is specific for some DRG neurons and that the expression seen by RT-PCR in other tissues may be leaky or low-level expression in some (or perhaps most) cells within those tissues. Retrospective analysis of the literature on neuronal-specific LAT expression shows that most of these studies compared LAT expression in neuroblastoma vs. fibroblast cell lines, and the few *in vivo* analyses relied primarily on reporter or *in situ* hybridization analyses. Therefore our results serve to confirm and extend these analyses to indicate that the pattern of abundant LAT expression is likely controlled at the level of different types of neurons, and not other viral functions.

Mapping of the transgene insert determined that in addition to the SV40 poly A sequence being deleted, a small portion of up to 132 bp of the 3' end of the transcript was also deleted. This deletion does not appear to affect the splice acceptor site since the stable LAT intron can be detected in the DRG by Northern blot.

The LAT transgene has no detectable effect on altering the outcome of an experimental infection of the transgenic mice. Experiments presented here examined the acute, establishment and reactivation phases of the HSV infection and in each of these phases there was no difference between the transgenic and non-transgenic mice. Additionally, infection of the transgenic mice with the 17Δ480 virus, a LAT mutant with reduced virulence, did not rescue the virulence phenotype. There were slight but

insignificant differences seen during the acute phase of infection and these differences will be re-evaluated by backcrossing the transgenic mouse into the Swiss-Webster background, which is more sensitive to HSV infection and then repeating the infection study. However, given the fact that the 17Δ480 virus is also restricted in replication in PC-12 cells in vitro, our favored hypothesis is that the transgenic mouse in the Swiss-Webster background will not rescue the virulence of 17Δ480.

On the whole, the data presented here suggest that, in the context of the HSV-1 genome, the LAT is functioning in *cis* to regulate or affect the DNA that transcribed it in order to modulate the genetically distinct virulence and reactivation phenotypes that map to this region. There are a number of possible mechanisms for this LAT function, including acting as a boundary element to prevent transcription of the surrounding acute transcripts or aiding modification of the chromatin structure to control transcription in this important regulatory region of the viral genome. Further studies with the transgenic mouse will investigate these potential regulation mechanisms.

APPENDIX A
MAP AND SEQUENCE OF THE pLAT/LAT PLASMID

The pLAT/LAT plasmid was generated to construct the transgene insert. A portion of the HSV-1 strain 17+ latency associated transcript (LAT) including the DraI site (corresponding to HSV-1 genome base pair 116,516) to the AatII site (corresponding to HSV-1 genome base pair 121,549) was ligated into a pBluescript backbone at the SmaI site. The SV40 poly A sequence from pNSE-Ex4 was removed using EcoRI and inserted at the XbaI site into the pBluescript plasmid containing the LAT sequence.

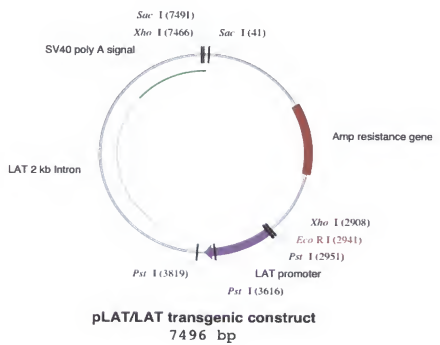

Figure A-1. Plasmid map of the HSV-1 LAT transgenic construct. This plasmid was linearized by digesting with Xho1 for injection into the mouse pronucleus as described in Chapter 2.

Presented here is the nucleotide sequence of pLAT/LAT plasmid used to generate the HSV-1 LAT transgenic mouse.

1 gatccactag ttctagagcg gccgccaccg cggtggagct ccagcttttg ttccctttag

61 tgagggttaa tttcgagctt ggcgtaatca tggtcatagc tgtttcctgt gtgaaattgt

121 tatccgctca caattccaca caacatacga gccggaagca taaagtgtaa agcctggggt

181 gcctaatgag tgagctaact cacattaatt gcgttgcgct cactgcccgc tttccagtcg

241 ggaaacctgt cgtgccagct gcattaatga atcggccaac gcgcggggag aggcggtttg

301 cgtattgggc gctcttccgc ttcctcgctc actgactcgc tgcgctcggt cgttcggctg

361 cggcgagcgg tatcagctca ctcaaaggcg gtaatacggt tatccacaga atcaggggat

421 aacgcaggaa agaacatgtg agcaaaaggc cagcaaaagg ccaggaaccg taaaaaggcc

481 gcgttgctgg cgtttttcca taggctccgc cccctgacg agcatcacaa aaatcgacgc

541 tcaagtcaga ggtggcgaaa cccgacagga ctataaagat accaggcgtt tcccctgga

601 agctccctcg tgcgctctcc tgttccgacc ctgccgctta ccggatacct gtccgccttt

661 ctcccttcgg gaagcgtggc gctttctcat agctcacgct gtaggtatct cagttcggtg

721 taggtcgttc gctccaagct gggctgtgtg cacgaacccc ccgttcagcc cgaccgctgc

781 gccttatccg gtaactatcg tcttgagtcc aacccggtaa gacacgactt atcgccactg

841 gcagcagcca ctggtaacag gattagcaga gcgaggtatg taggcggtgc tacagagttc

901 ttgaagtggt ggcctaacta cggctacact agaaggacag tatttggtat ctgcgctctg

961 ctgaagccag ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc

1021 gctggtagcg gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa aaaaggatct

1081 caagaagatc ctttgatctt ttctacgggg tctgacgctc agtggaacga aaactcacgt

1141 taagggattt tggtcatgag attatcaaaa aggatcttca cctagatcct tttaaattaa

1201 aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa cttggtctga cagttaccaa

1261 tgcttaatca gtgaggcacc tatctcagcg atctgtctat ttcgttcatc catagttgcc

1321 tgactccccg tcgtgtagat aactacgata cgggagggct taccatctgg ccccagtgct

1381 gcaatgatac cgcgagaccc acgctcaccg gctccagatt tatcagcaat aaaccagcca

1441 gccggaaggg ccgagcgcag aagtggtcct gcaactttat ccgcctccat ccagtctatt

1501 aattgttgcc gggaagctag agtaagtagt tcgccagtta atagtttgcg caacgttgtt

1561 gccattgcta caggcatcgt ggtgtcacgc tcgtcgtttg gtatggcttc attcagctcc

1621 ggttcccaac gatcaaggcg agttacatga tcccccatgt tgtgcaaaaa agcggttagc

1681 tccttcggtc ctccgatcgt tgtcagaagt aagttggccg cagtgttatc actcatggtt

1741 atggcagcac tgcataattc tcttactgtc atgccatccg taagatgctt ttctgtgact

1801 ggtgagtact caaccaagtc attctgagaa tagtgtatgc ggcgaccgag ttgctcttgc

1861 ccggcgtcaa tacgggataa taccgcgcca catagcagaa ctttaaaagt gctcatcatt

1921 ggaaaacgtt cttcggggcg aaaactctca aggatcttac cgctgttgag atccagttcg

1981 atgtaaccca ctcgtgcacc caactgatct tcagcatctt ttactttcac cagcgtttct

2041 gggtgagcaa aaacaggaag gcaaaatgcc gcaaaaaagg gaataagggc gacacggaaa

2101 tgttgaatac tcatactctt cctttttcaa tattattgaa gcatttatca gggttattgt

2161 ctcatgagcg gatacatatt tgaatgtatt tagaaaaata aacaaatagg ggttccgcgc

2221 acatttcccc gaaaagtgcc acctaaattg taagcgttaa tattttgtta aaattcgcgt

2281 taaatttttg ttaaatcagc tcatttttta accaataggc cgaaatcggc aaaatccctt

2341 ataaatcaaa agaatagacc gagatagggt tgagtgttgt tccagtttgg aacaagagtc

2401 cactattaaa gaacgtggac tccaacgtca aagggcgaaa aaccgtctat cagggcgatg

2461 gcccactacg tgaaccatca ccctaatcaa gttttttggg gtcgaggtgc cgtaaagcac

2521 taaatcggaa ccctaaaggg agcccccgat ttagagcttg acggggaaag ccggcgaacg

2581 tggcgagaaa ggaagggaag aaagcgaaag gagcgggcgc tagggcgctg gcaagtgtag

2641 cggtcacgct gcgcgtaacc accacacccg ccgcgcttaa tgcgccgcta cagggcgcgt

2701 cccattcgcc attcaggctg cgcaactgtt gggaagggcg atcggtgcgg gcctcttcgc

2761 tattacgcca gctggcgaaa gggggatgtg ctgcaaggcg attaagttgg gtaacgccag

2821 ggttttccca gtcacgacgt tgtaaaacga cggccagtga attgtaatac gactcactat

2881 agggcgaatt gggtaccggg ccccccctcg aggtcgacgg tatcgataag cttgatatcg

2941 aattcctgca gcccaaataa accaatgtcg gaataaacaa acacaaacac ccgcgacggg

3001 gggacggagg ggacggaggg agggggtgac gggggacggg aacagacaca aaaacaacca

3061 caaaaaacaa ccacccaccg acacccccac cccagtctcc tcgccttctc ccacccaccc

3121 cacgccccca ctgagcccgg tcgatcgacg agcacccccg cccacgcccc cgcccctgcc

3181 ccggcgaccc ccggcccgca cgatcccgac aacaataaca accccaacgg aaagcggcgg

3241 ggtgttgggg gaggcgagga acaaccgagg ggaacggggg atggaaggac gggaagtgga

3301 agtcctgata cccatcctac acccccctgc cttccaccct ccggcccccc gcgagtccac

3361 ccgccggccg gctaccgaga ccgaacacgg cggccgccgc agccgccgca gccgccgccg

3421 acaccgcaga gccggcgcgc gcactcacaa gcggcagagg cagaaaggcc cagagtcatt

3481 gtttatgtgg ccgcgggcca gcagacggcc cgcgacaccc ccccccgcc cgtgtgggta

3541 tccggccccc cgccccgcgc cggtccatta agggcgcgcg tgcccgcgag atatcaatcc

3601 gttaagtgct ctgcagacag gggcaccgcg cccggaaatc cattaggccg cagacgagga

3661 aaataaaatt acatcaccta cccacgtggt gctgtggcct gtttttgctg cgtcatctca

3721 gcctttataa aagcgggggc gcggccgtgc cgatcgcggg tggtgcgaaa gactttccgg

3781 gcgcgtccgg gtgccgcggc tctccgggcc cccctgcagc cggggcggcc aaggggcgtc

3841 ggcgacatcc tccccctaag cgccggccgg ccgctggtct gttttttcgt tttccccgtt

3901 tcgggggtgg tgggggttgc ggtttctgtt tctttaaccc gtctgggggtg tttttcgttc

3961 cgtcgccgga atgtttcgtt cgtctgtccc ctcacggggc gaaggccgcg tacggcccgg

4021 gacgaggggc ccccgaccgc ggcggtccgg gccccgtccg gacccgctcg ccggcacgcg

4081 acgcgaaaaa ggccccccgg aggcttttcc gggttcccgg cccggggcct gagatgaaca

4141 ctcggggtta ccgccaacgg ccggcccccg tggcggcccg gcccgggccc ccggcggacc

4201 caaggggccc cggcccgggg ccccacaacg gcccggcgca tgcgctgtgg ttttttttte

4261 ctcggtgttc tgccgggctc catcgccttt cctgttctcg cttctccccc ccccttctt

4321 cacccccagt accctcctcc ctcccttcct ccccgttat cccactcgtc gagggcgccc

4381 cggtgtcgtt caacaaagac gccgcgtttc caggtaggtt agacacctgc ttctccccaa

4441 tagaggggggg ggacccaaac gacaggggge gccccagagg ctaaggtcgg ccacgccact

4501 cgcgggtggg ctcgtgttac agcacaccag cccgttcttt tcccccctc ccacccttag

4561 tcagactctg ttacttaccc gtccgaccac caactgcccc cttatctaag ggccggctgg

4621 aagaccgcca gggggtcggc cggtgtcgct gtaacccccc acgccaatga cccacgtact

4681 ccaagaaggc atgtgtccca ccccgcctgt gtttttgtgc ctggctctct atgcttgggt

4741 cttactgcct gggggggggg agtgcggggg agggggggtg tggaaggaaa tgcacggcgc

4801 gtgtgtaccc cccctaaagt tgttcctaaa gcgaggatac ggaggagtgg cgggtgccgg

4861 gggaccgggg tgatctctgg cacgcggggg tgggaagggt cgggggaggg ggggatggag

4921 taccggccca cctggccgcg cgggtgcgcg tgcctttgca caccaacccc acgtccccg

4981 gcggtctcta agaagcaccg ccccccctcc ttcataccac cgagcatgcc tgggtgtggg

5041 ttggtaacca acacgcccat ccctcgtct cctgtgattc tctggctgca ccgcattctt

5101 gttttctaac tatgttcctg tttctgtctc ccccccccc acccctccgc cccacccccc

5161 aacacccacg tctgtggtgt ggccgacccc cttttgggcg ccccgtcccg ccccgccacc

5221 cctcccatcc tttgttgccc tatagtgtag ttaaccccc ccgccctttg tggcggccag

5281 aggccaggtc agtccgggcg ggcaggcgct cgcggaaact taacacccac acccaaccca

5341 ctgtggttct ggctccatgc cagtggcagg atgctttcgg ggatcggtgg tcaggcagcc

5401 cgggccgcgg ctctgtggtt aacaccagag cctgcccaac atggcacccc cactcccacg

5461 cacccccact cccacgcacc cccactccca cgcaccccca ctcccacgca cccccactcc

5521 cacgcacccc cactcccacg cacccccact cccacgcacc cccactccca cgcaccccca

5581 ctcccacgca tccccgcgat acatccaaca cagacaggga aaagatacaa aagtaaacct

5641 ttatttccca acagacagca aaaatcccct gagtttttt ttattagggc caacacaaaa

5701 gacccgctgg tgtgtggtgc ccgtgtcttt cacttttccc ctccccgaca cggattggct

5761 ggtgtagtgg gcgcggccag agaccaccca gcgcccgacc ccccctccc cacaaacacg

5821 gggggcgtcc cttattgttt tccctcgtcc cgggtcgacg ccccctgctc cccggaccac

5881 gggtgccgag accgcaggct gcggaagtcc agggcgccca ctagggtgcc ctggtcgaac

5941 agcatgttcc ccacgggggt catccagagg ctgttccact ccgacgcggg ggccgtcggg

6001 tactcggggg gcatcacgtg gttacccgcg gtctcgggga gcagggtgcg gcggctccag

6061 ccggggaccg cggcccgcag ccgggtcgcc atgtttcccg tctggtccac caggaccacg

6121 tacgccccga tgttccccgt ctccatgtcc aggatgggca ggcagtcccc cgtgatagtc

6181 ttgttcacgt aaggcgacag ggcgaccacg ctagagaccc ccgagatggg caggtagcgc

6241 gtgaggccgc ccgcggggac ggccccggaa gtctccgcgt ggcgcgtctt ccgggcacac

6301 ttcctcggcc cccgcggccc agaagcagcg cgggggccga gggaggtttc ctcttgtctc

6361 cctcccaggg caccgacggc cccgcccgag gaggcggaag cggaggagga cgcggccccg

6421 gcggcggaag aggcggcccc cgcgggggtc ggggccgagg aggaagaggc agaggaggaa

6481 gaggcggagg ccgccgaggg ggggatcaat tcagctgagc gccggtcgct accattacca

6541 gttggtctgg tgtcaaaaat aataataacc gggcaggggg gatctgcatg gatcgatcca

6601 gacatgataa gatacattga tgagtttgga caaaccacaa ctagaatgca gtgaaaaaaa

6661 tgctttattt gtgaaatttg tgatgctatt gctttatttg taaccattat aagctgcaat

6721 aaacaagtta acaacaacaa ttgcattcat tttatgtttc aggttcaggg ggaggtgtgg

6781 gaggtttttt aaagcaagta aaacctctac aaatgtggta tggctgatta tgatctctag

6841 tcaaggcact atacatcaaa tattccttat taaccccttt acaaattaaa aagctaaagg

6901 tacacaattt ttgagcatag ttattaatag cagacactct atgcctgtgt ggagtaagaa

6961 aaaacagtat gttatgatta taactgttat gcctacttat aaaggttaca gaatattttt

7021 ccataatttt cttgtatagc agtgcagctt tttcctttgt ggtgtaaata gcaaagcaag

7081 caagagttct attactaaac acagcatgac tcaaaaaact tagcaattct gaaggaaagt

7141 ccttggggtc ttctaccttt ctcttctttt ttggaggagt agaatgttga gagtcagcag

7201 tagcctcatc atcactagat ggcatttctt ctgagcaaaa caggttttcc tcattaaagg

7261 cattccacca ctgctcccat tcatcagttc cataggttgg aatctaaaat acacaaacaa

7321 ttagaatcag tagtttaaca cattatacac ttaaaaattt tatatttacc ttagagcttt

7381 aaatctctgt aggtagtttg tccaattatg tcacaccaca gaagtaaggt tccttcacaa

7441 agatcctcta gcgataccgt cgacctcgag gggggcccg gtaccgagct cgaatt

APPENDIX B
PCR PRIMER SEQUENCES

Polymerase chain reaction (PCR) was used in the experiments described in this dissertation for a number of quantifications and identifications. The complete list of primers used for these reactions is presented here in tabular form for easy reference.

Table B-1. Conventional PCR Primers and Locations

Primer Name	Sequence	Genome Location	PCR Conditions[1]
Promar 1	GCA CGA TCC CGA CAA CAA TAA CAA C	118,246-118,270	94°, 55°, 72°
Promar 2	ACT TCC ACT TCC CGT CCT TCC ATC C	118,327-118,351	94°, 55°, 72°
DB60	CGG CGA CAT CCT CCC CCT AAG C	118,888-118,910	94°, 55°, 72°
DB61	GAC AGA CGA ACG AAA CAT TCC G	118,994-119,016	94°, 55°, 72°
M int 1	GAC ACG CAT TGG CTG GTG TAG TGG G	120,795-120,819	94°, 55°, 72°
M int 2	ACG AGG GAA AAC AAT AAG GGA CGC C	120,872-120,898	94°, 55°, 72°
M2 probe up	AGA CCC GCT GGT GG TGG TG	120,748-120,767	94°, 55°, 72°
M2 probe down	GAT GCC CCC CGA GTA CCC GA	121,044-121,063	94°, 55°, 72°
AG 29	CGG GTA CTC GGG GGG CA	121,044-121,061	94°, 55°, 72°
AG 30	CTC GGG GGT CTC TAG CGT GG	121,252-121,272	94°, 55°, 72°
AG 31	CGC CTC TTC CTC CTC TGC CT	121,513-121,533	94°, 68°, 72°

[1] All PCR conditions are one cycle for 3 minutes followed by 30 cycles for 1 minute.

Table B-2. Real Time PCR Primer and Probe Sequences

Primer Name	**Sequence**
XIST forward	GCT CTT AAA CTG AGT GGG TGT TCA
XIST reverse	GTA TCA CGC AGA AGC CAT AAT GG
XIST probe	ACG CGG GCT CTC CA
5'LAT forward	GGC TCC ATC GCC TTT CCT
5'LAT reverse	AAG GGA GGG AGG AGG GTA CTG
5'LAT probe	TCT CGC TTC TCC CC
LPRO forward	CAA TAA CAA CCC CAA CGG AAA GC
LPRO reverse	TCC ACT TCC CGT CCT TCC AT
LPRO probe	TCC CCT CGG TTG TTC C
POL forward	AGA GGG ACA TCC AGG ACT TTG T
POL reverse	CAG GCG CTT GTT GGT GTA C
POL probe	ACC GCC GAA CTG AGC A

APPENDIX C
EXPRESSION OF THE LAT TRANSGENE IS NOT AGE DEPENDENT

The expression profile for the LAT transgenic mouse was presented in Chapter 3. Due to the large amount of data generated by studying the expression profile in four age groups of mice, only a portion of the age related expression data was presented. The complete age related expression profile for the transgenic mouse is presented here.

For each tissue RNA was extracted and reverse transcribed as described in the materials and methods, chapter 3. RT-PCR reactions were performed using real time PCR for the 5' LAT exon or 18s RNA and compared to a standard curve generated by known copies of plasmid DNA.

The LAT expression in transgenic neural tissues for the four age groups, 1 day, 1 month, 2 months, and 18 months old, is graphed in figure C-1. By ANOVA there was no statistical difference between the age groups for each tissue, hypothalamus (P= 0.1744), cerebellum (P= 0.7774), cortex (P= 0.7537), olfactory bulb (P= 0.5967), spinal cord (P= 0.3303), dorsal root ganglia (P= 0.6908), trigeminal ganglia (P= 0.4050) when represented per gram of tissue.

Figure C-2 illustrates the expression data for non-neural tissues at the four age groups. There was no statistically significant difference between the ages of any of the tissues tested. Skin (P= 0.1728), foot (P= 0.0657), heart (P= 0.1623), kidney (P= 0.5630), lung (P= 0.2311), eye (P= 0.6017), liver (P= 0.3415), spleen (P= 0.5750), and intestine (P= 0.6113).

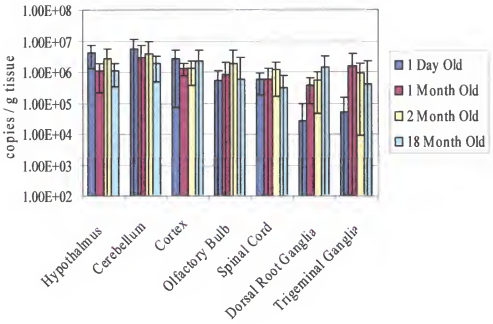

Figure C-1. Expression of the LAT transgene in neural tissues. Expression is represented as LAT copies per gram of tissue. n = 4.

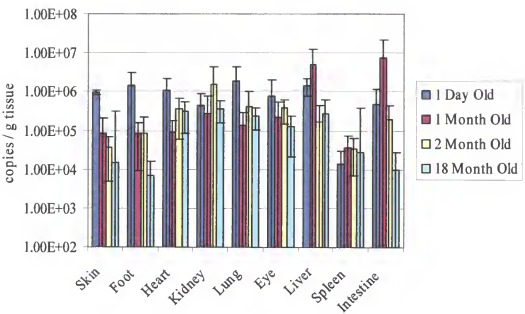

Figure C-2. Expression of the LAT transgene in non-neural tissues. Expression is represented as LAT copies per gram of tissue. n = 4.

As discussed in chapter 3, comparison of different tissues on a per weight basis does not take into consideration the different sizes and densities of each cell type. Thus, this representation is not on a per cell basis. To address this issue, tissue expression was compared normalized to levels of 18s RNA which was considered to be constant in all cells (Thellin et al., 1999).

As for the per weight analysis, tissues were divided into neural tissues (figure C-3) and non-neural tissues (figure C-4). The neural tissues when normalized to 18s RNA exhibited no significant difference between the amount of expression at the four age groups. Hypothalamus (P= 0.7320), cerebellum (P= 0.6499), cortex (P= 0.2609), olfactory bulb (P= 0.4991), spinal cord (P= 0.2859), dorsal root ganglia (P= 0.4803), and trigeminal ganglia (P= 0.2429).

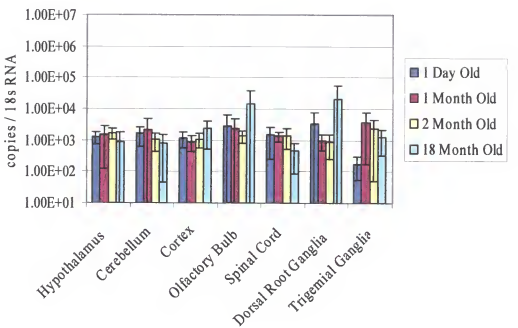

Figure C-3. Expression of the LAT transgene in neural tissues represented as copies per 18s RNA.

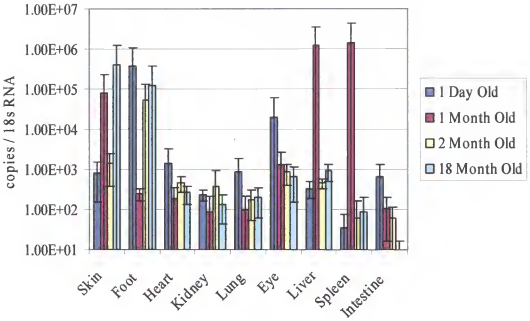

Figure C-4. Expression of the LAT transgene in non-neural tissues represented as copies per 18s RNA.

The non-neural tissues were not statistically different between the age groups when normalized to 18s RNA. Skin (P= 0.5305), foot (P= 0.5707), heart (P= 0.2801), kidney (P= 0.5286), lung (P= 0.1606), eye (P=0.4662), liver (P= 0.3247), spleen (P= 0.4737), and intestine (P= 0.2770).

Among all of the tissues tested at the four age groups, only the foot was close to having a significant age dependent expression pattern. If more mice were sampled it is possible that the expression in the foot would be age dependent on a per weight basis. Due to the fact that the expression in the foot when compared on per 18s RNA basis was not significant, there is probably no scientific merit to difference in expression in the foot especially since the foot consists of many different cell types including the bone and muscles. Thus, there is no age dependent expression of the LAT transgene in these tissues.

LIST OF REFERENCES

Ahmed, M., M. Lock, C. G. Miller and N. W. Fraser (2002). "Regions of the herpes simplex virus type 1 latency-associated transcript that protect cells from apoptosis in vitro and protect neural cells in vivo." Journal of Virology 76(2): 717-729

Alvira, M. R., W. F. Goins, J. B. Cohen and J. Glorioso (1999). "Genetic studies exposing the splicing events involved in herpes simplex virus type 1 latency-associated transcript production during lytic and latent infection." Journal of Virology 73(5): 3866-3876.

Anderson, J. M. and M. W. N. Nicholls (1972). "Herpes encephalitis in pregnancy." British Medical Journal 1(800): 632.

Berthomme, H., J. Lokensgard, L. Yang, T. Margolis and L. T. Feldman (2000). "Evidence for a bidirectional element located downstream from the herpes simplex virus type 1 latency-associated promoter that increases its activity during latency." Journal of Virology 74(8): 3613-3622.

Berthomme, H., J. Thomas, P. Texier, A. Epstein and L. T. Feldman (2001). "Enhancer and long-term expression functions of herpes simplex virus type 1 latency-associated promoter are both located in the same region." Journal of Virology 75(9): 4386-4393.

Bhattacharjee, P. S., R. K. Tran, M. E. Myles, K. Maruyama, A. Mallakin, D. C. Bloom and J. M. Hill (2003). "Overlapping subdeletions within a 348-bp in the 5' exon of the LAT region that facilitates epinephrine-induced reactivation of HSV-1 in the rabbit ocular model do not further define a functional element." Virology 312(1): 151-158.

Bloom, D. C., G. B. Devi-Rao, J. M. Hill, J. G. Stevens and E. K. Wagner (1994). "Molecular analysis of herpes simplex virus type 1 during epinephrine induced reactivation of latently infected rabbits in vivo." Journal of Virology 68(3): 1283-1292.

Bloom, D. C., J. M. Hill, E. K. Wagner, L. T. Feldman and J. G. Stevens (1996). "A 348-bp region in the latency associated transcript facilitates herpes simplex virus type 1 reactivation." Journal of Virology 70: 2449-2459.

Burton, E. A., C. S. Hong and J. C. Glorioso (2003). "The stable 2.0-kilobase intron of the herpes simplex virus type 1 latency-associated transcript does not function as an antisense repressor of ICP0 in nonneuronal cells." Journal of Virology 77(6): 3516-3530.

Cai, W. and P. Schaffer (1992). "Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA." Journal of Virology 66: 2904-2915.

Chen, S. H., M. F. Kramer, P. A. Schaffer and D. M. Cohen (1997). "A viral function represses accumulation of transcripts from productive-cycle genes in mouse ganglia latently infected with herpes simplex virus." Journal of Virology 71: 5878-5884.

Chen, Y. T., Y. H. Wang, Y. Y. Cheng and S. L. Hung (2003). "Direct binding of herpes simplex virus type 1 virions to complement C3." Viral Immunology 16(3): 347-355.

Coffin, R. S. and M. S. Thomas (1998). "The herpes simplex virus 2 kb latency-associated transcript (LAT) leader sequence allows efficient expression of downstream proteins which is enhanced in neuronal cells: possible function of LAT ORFs." Journal of General Virology 79: 3019-3026.

Colgin, M. A., R. L. Smith and C. L. Wilcox (2001). "Inducible cyclic AMP early repressor produces reactivation of latent herpes simplex virus type 1 in neurons in vitro." Journal of Virology 75(6): 2912-2920.

Davido, D. J., W. F. von Zagorski, G. G. Maul and P. A. Schaffer (2003). "The differential requirement for cyclin-dependent kinase activities distinguishes two functions of herpes simplex virus type 1 ICP0." Journal of Virology 77(23): 12603-12616.

Dobson, A. T., T. P. Margolis, W. A. Gomes and L. T. Feldman (1995). "In vivo deletion analysis of the herpes simplex virus type 1 latency associated transcript promoter." Journal of Virology 69(4): 2264-2270.

Drolet, B. S., G. C. Perng, J. Cohen, S. M. Slanina, A. Yukht, A. B. Nesburn and S. L. Wechsler (1998). "The region of the herpes simplex virus type 1 LAT gene involved in spontaneous reactivation does not encode a functional protein." Virology 242: 221-232.

Ellis, J., P. Pasceri, K. C. Tan-Un, X. Wu, A. Harper, P. Fraser and F. Grosveld (1997). "Evaluation of beta-globin gene therapy constructs in single copy transgenic mice." Nucleic Acids Research 25:1296-1302.

Ellison, A. R., L. Yang, C. C. Voytek and T. Margolis (2000). "Establishment of latent herpes simplex virus type 1 infection in resistant, sensitive, and immunodeficient mouse strains." Virology 268: 17-28.

Farrell, M. J., A. T. Dobson and L. T. Feldman (1991). "Herpes simplex virus latency-associated transcript is a stable intron." Proceedings of the National Academy of Sciences 88: 790-794.

Gary, L.W., J.M. Loutsch, J. M. Hill, E.K. Wagner and D.C. Bloom. In preparation. "Identification of a virulence function in herpes simplex virus type 1 mapping to the latency-associated transcript locus."

Gesser, R. M., T. Valyi-Nagy and N. Fraser (1994). "Restricted herpes virus type 1 gene expression within sensory neurons in the absence of functional B and T lymphocytes." Virology 200: 791-795.

Goins, W. F., L. R. Sternberg, K. D. Croen, P. R. Krause, R. L. Hendricks, D. J. Fink, S. E. Straus, M. Levine and J. C. Glorioso (1994). "A novel latency-active promoter is contained within the herpes simplex virus type 1 UL flanking repeats." Journal of Virology 68(4): 2239-2252.

Gordon, J. W., G. A. Scangos, D. J. Plotkin, J. A. Barbosa and F. H. Ruddle (1980). "Genetic transformation of mouse embryos by microinjection of purified DNA." Proceedings of the National Academy of Sciences 77(12): 7380-7384.

Gressens, P. and J. R. Martin (1994). "In situ polymerase chain reaction: localization of HSV-2 DNA sequences in infections of the nervous system." Journal of Virology Methods 46: 61-83.

Grunewald, K., P. Desai, D. C. Winkler, J. B. Heymann, D. M. Belnap, W. Baumeister and A. L. Steven (2003). "Three-dimensional structure of herpes simplex virus from cryo-electron tomography." Science 302: 1396-1398.

Halford, W. P. and P. A. Schaffer (2001). "ICP0 is required for efficient reactivation of herpes simplex virus type 1 from neuronal latency." Journal of Virology 75(7): 3240-3249.

Halford, W. P., C. D. Kemp, J. A. Isler, D. J. Davido and P. Schaffer (2001). "ICP0, ICP4, or VP16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells." Journal of Virology 75(13): 6143-6153.

Hill, J. M., J. B. Dudley, Y. Shimomura and H. E. Kaufman (1986). "Quantitation and kinetics of adrenergic induced HSV-1 ocular shedding." Current Eye Research 5: 241-246.

Hill, J. M., J. B. Maggioncalda, H. H. Garza, Y.-H. Su, N. W. Fraser and T. M. Block (1996). "In vivo epinephrine reactivation of ocular herpes simplex type 1 (HSV-1) in the rabbit is correlated to a 370 base pair region located between the promoter and the 5' end of the 2.0 kb latency-associated transcript (LAT)." Journal of Virology 70: 7270-7274.

Hogan, B., F. Constantini and E. Lacy (1986). Manipulating the mouse genome. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.

Jackson, S. A. and N. A. DeLuca (2003). "Relationship of herpes simplex virus genome configuration to productive and persistent infections." Proceedings of the National Academy of Sciences 100(13): 7871-7876.

Jarman, R. G., E. K. Wagner and D. C. Bloom (1999). "LAT expression during an acute HSV infection in the mouse." Virology 262: 384-397.

Jarman, R. G., J. M. Loutsch, G. B. Devi-Rao, M. E. Marquart, M. P. Banaszak, X. Zheng, J. M. Hill, E. K. Wagner and D. C. Bloom (2002). "The region of the HSV-1 latency-associated transcript required for epinephrine-induced reactivation in the rabbit does not include the 2.0 kb intron." Virology 292: 59-69.

Kastrukoff, L. F., A. S. Lau and M. L. Puterman (1986). "Genetics of natural resistance to herpes simplex virus type 1 latent infection of the peripheral nervous system in mice." Journal of General Virology 67: 613-621.

Kenny, J. J., F. C. Krebs, H. T. Hartle, A. E. Gartner, B. Chatton, J. M. Leiden, J. P. Hoeffler, P. C. Weber and B. Wigdahl (1994). "Identification of a second ATF/CREB-like element in the herpes simplex virus type 1 (HSV-1) latency-associated transcript (LAT) promoter." Virology 200: 220-235.

Koelle, D. M. and L. Corey (2003). "Recent progress in herpes simplex virus immunobiology and vaccine research." Clinical Microbiology Reviews 16(1): 96-113.

Kubat, N. J., R. K. Tran, P. McAnany and D. Bloom (2004). "Specific histone tail modification and not DNA methylation is a determinant of HSV-1 latent gene expression." Journal of Virology 78(3): 1139-1149.

Levine, M., A. Krikos, J. C. Glorioso and F. L. Homa (1990). "Regulation of expression of the glycoprotein genes of herpes simplex virus type 1 (HSV-1)." Advances in Experimental Medicine and Biology 278: 151-164.

Loiacono, C. M., R. Myers and W. J. Mitchell (2002). "Neurons differentially activate the herpes simplex virus type 1 immediate-early gene ICP0 and ICP27 promoters in transgenic mice." Journal of Virology 76(5): 2449-2459.

Lokensgard, J. R., H. Berthomme and L. T. Feldman (1997). "The latency-associated promoter of herpes simplex virus type 1 requires a region downstream of the transcription start site for long-term expression during latency." Journal of Virology 71(9): 6714-6719.

Lopez, C. (1975). "Genetics of natural resistance to herpes simplex virus infections in mice." Nature 258: 152-153.

Mackem, S. and B. Roizman (1982). "Differentiation between alpha promoter and regulator regions of herpes simplex virus 1: the functional domains and sequence of a movable alpha regulator." Proceedings of the National Academy of Sciences 79(16): 4917-4921.

Mador, N., E. Braun, H. Haim, I. Ariel, A. Panet and I. Steiner (2003). "Transgenic mouse with the herpes simplex virus type 1 latency-associated gene: expression and function of the transgene." Journal of Virology 77(23): 12421-12429.

Maggioncalda, J., A. Mehta, Y. H. Su, N. W. Fraser and T. M. Block (1996). "Correlation between herpes simplex virus type 1 rate of reactivation from latent infection and the number of infected neurons in trigeminal ganglia." Virology 225: 72-81.

Margolis, T. P., F. Sedarati, A. T. Dobson, L. T. Feldman and J. G. Stevens (1992). "Pathways of viral gene expression during acute neuronal infection with HSV-1." Virology 189(1): 150-160.

Margolis, T. P., D. C. Bloom, A. T. Dobson, L. T. Feldman and J. G. Stevens (1993). "LAT promoter activity decreases dramatically during the latent phase of ganglionic infection with HSV." Virology 197(2): 585-592.

Mitchell, W. J. (1995). "Neurons differentially control expression of a herpes simplex virus type 1 immediate-early promoter in transgenic mice." Journal of Virology 69(12): 7942-50.

Nomura, T. (1997). "Practical development of genetically engineered animals as human disease models." Laboratory Animal Science 47(2): 113-117.

Nicosia, M., S. L. Deshmane, J. M. Zaboloty, T. Valyi-Nagy and N. Fraser (1993). "Herpes simplex virus type 1 latency-associated transcript (LAT) promoter deletion mutants can express a 2-kilobase transcript mapping to the LAT region." Journal of Virology 67(12): 7276-7283.

Perng, G.-C., E. C. Dunkel, P. A. Geary, S. M. Slanina, H. Ghiasi, R. Kaiwar, A. B. Nesburn and S. L. Wechsler (1994). "The latency-associated transcript gene of herpes simplex virus type 1 (HSV-1) is required for efficient in vivo spontaneous reactivation of HSV-1 from latency." Journal of Virology 68(12): 8045-8055.

Perng, G. C., S. M. Slanina, A. Yukht, B. S. Drolet, W. Keleher, Jr., H. Ghiasi, A. B. Nesburn and S. L. Wechsler (1999). "A herpes simplex virus type 1 latency-associated transcript mutant with increased virulence and reduced spontaneous reactivation." Journal of Virology 73(2): 920-929.

Perng, G. C., C. Jones, J. Ciacci-Zanella, M. Stone, G. Henderson, A. Yukht, S. M. Slanina, F. M. Hofman, H. Ghiasi, A. B. Nesburn and S. L. Wechsler (2000). "Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript." Science 287(5457): 1500-1503.

Perng, G. C., B. Maguen, L. Jin, K. R. Mott, J. Kurylo, L. BenMohamed, A. Yukht, N. Osorio, A. B. Nesburn, G. Henderson, M. Inman, C. Jones and S. L. Wechsler (2002). "A novel herpes simplex virus type 1 transcript (AL-RNA) antisense to the 5' end of the latency associated transcript produces a protein in infected rabbits." Journal of Virology 76(16): 8003-8010.

Perry, L. J. and D. J. McGeoch (1988). "The DNA sequences of the long repeat region and adjoining parts of the long unique region in the genome of herpes simplex virus type 1." Journal of General Virology 69(11): 2831-2846.

Qiagen, Inc. (2003). Qiagen Product Guide. Valencia, CA.

Rall, G. F., L. Mucke and M. B. A. Oldstone (1995). "Consequences of cytotoxic T lymphocyte interaction with major histocompatibility complex class 1-expressing neurons in vivo." Journal of Experimental Medicine 182: 1201-1212.

Rodahl, E. and J. G. Stevens (1992). "Differential accumulation of herpes simplex virus type 1 latency-associated transcripts in sensory and autonomic ganglia." Virology 189(1): 385-388.

Roizman, B. and A. E. Sears (1996). Herpes Simplex Viruses and Their Replication. Virology. B. N. Fields. New York, Raven Press, Ltd., 2: 1795-1841.

Sawtell, N. M. and R. L. Thompson (1992). "Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia." Journal of Virology 66(4): 2150-2156.

Shibata, S. and J. T. Lee (2003). "Characterization and quantitation of differential *Tsix* transcripts: implications for *Tsix* function." Human Molecular Genetics 12(2): 125-136.

Sippel, A. E., H. Saueressig, M. C. Huber, N. Faust and C. Bonifer (1997). Insulation of transgenes from chromosomal position effects. Transgenic Animals Generation and Use. L. M. Houdebine. Amsterdam, OPA.

Smith, C. A., P. Bates, R. Rivera-Gonzalez, B. Gu and N. A. DeLuca (1993). "ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB." Journal of Virology 67(8): 4676-4687.

Soares, K., D. Hwang, R. Ramakrishnan, M. Schmidt, D. J. Fink and J. Glorioso (1996). "*Cis*-acting elements involved in transcriptional regulation of the herpes simplex virus type 1 latency-associated promoter 1 (LAP1) *in vitro* and *in vivo*." Journal of Virology 70(8): 5384-5394.

Speck, P. G. and A. Simmons (1991). "Divergent molecular pathways of productive and latent infection with a virulent strain of herpes simplex virus type 1." Journal of Virology 65(8): 4001-4005.

Spivack, J. G., G. M. Woods and N. W. Fraser (1991). "Identification of a novel latency-specific splice donor signal within the herpes simplex virus type 1 2.0-kilobase latency-associated transcript (LAT): translation inhibition of LAT open reading frames by the intron within the 2.0-kilobase LAT." Journal of Virology 65: 6800-6810.

Thellin, O., W. Zorzi, B. Lakaye, B. De Borman, B. Coumans, G. Hennen, T. Grisar, A. Igout and E. Heinen (1999). "Housekeeping genes as internal standards: uses and limits." Journal of Biotechnology 75: 291-295.

Thomas, S. K., G. Gough, D. S. Latchman and R. S. Coffin (1999). "Herpes simplex virus latency-associated transcript encodes a protein which greatly enhances growth, can compensate for deficiencies in immediate early gene expression, and is likely to function during reactivation from virus latency." Journal of Virology 73(8): 6618-6625.

Thomas, D. L., M. Lock, J. Zaboloty, B. R. Mohan and N. Fraser (2002). "The 2-kilobase intron of herpes simplex virus type 1 latency-associated transcript has a half-life of approximately 24 hours in SY5Y and COS-1 cells." Journal of Virology 76(2): 532-540.

Thompson, R. L. and N. M. Sawtell (2001). "Herpes simplex virus type 1 latency-associated transcript gene promotes neuronal survival." Journal of Virology 75(14): 6660-6675.

Voncken, J. W. (2003). Genetic modification of the mouse. Transgenic Mouse Methods and Protocols. M. H. Hofker and J. van Deursen. Totowa, NJ, Humana Press Inc.

Wagner, E. K. (1991). Herpesvirus Transcription and its Regulation. Boca Raton, CRC Press.

Wagner, E. K. and D. C. Bloom (1997). "Experimental investigation of herpes simplex virus latency." Clinical Microbiology Reviews 10(3): 419-443.

Wagner, E. K., M. D. Petroski, N. T. Pande, P. Leiu and M. K. Rice (1998). "Analysis of factors influencing the kinetics of herpes simplex virus transcript expression utilizing recombinant virus." Methods 16: 105-116.

Wang, K., L. Pesnicak and S. E. Straus (1997). "Mutations in the 5' end of the herpes simplex virus type 2 latency-associated transcript (LAT) promoter affect LAT expression *in vivo* but not the rate of spontaneous reactivation of genital herpes." Journal of Virology 71(10): 7903-7910.

Wang, K., L. Pesnicak, E. Guancial, P. Krause and S. E. Straus (2001). "The 2.2-kilobase latency-associated transcript of herpes simplex virus type 2 does not modulate viral replication, reactivation, or establishment of latency in transgenic mice." Journal of Virology 75(17): 8166-8172.

Ward, P. L. and B. Roizman (1998). Evasion and obstruction: the central strategy of the interaction of human herpesviruses with host defenses. Herpesviruses and Immunity. M. P.G., H. M. Friedman and M. Bendinelli. New York, Plenum Press.

Weir, J. P. (2001). "Regulation of herpes simplex virus gene expression." Gene 271: 117-130.

Whitley, R. J. (1990). Herpes Simplex Viruses. Virology. B. N. Fields. New York, Raven Press, Ltd., 2: 1843-1887.

Whitley, R. J. (1996). Herpes Simplex Viruses. Fields Virology. P. M. Howley. New York, Raven Press, Ltd., 2: 2297-2342.

Wilkie, T. M., R. L. Brinster and R. D. Palmiter (1986). "Germline and somatic mosaicism in transgenic mice." Developmental Biology 118: 9-18.

Wu, T.-T., Y.-H. Su, T. Block and J. M. Taylor (1998). "Atypical splicing of the latency-associated transcripts of herpes simplex type 1." Virology 243: 140-149.

Yang, L., C. C. Voytek and T. P. Margolis (2000). "Immunohistochemical analysis of primary sensory neurons latently infected with herpes simplex virus type 1." Journal of Virology 74(1): 209-217.

Zwaagstra, J. C., J. C. Ghiasi, S. M. Slanina, A. B. Nesburn, S. C. Wheatley, K. Lillycrop, J. Wood, D. S. Latchman, K. Patel and S. L. Wechsler (1990). "Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron-derived cells: evidence for neuron specificity and for a large LAT transcript." Journal of Virology 64: 5019-5028.

BIOGRAPHICAL SKETCH

Anne Gussow was born in Pottsville, Pennsylvania and graduated from Pottsville Area High School. She received her B.S. degree in biology from Susquehanna University in Selinsgrove, PA. She then went on to do research at the Pennsylvania State University College of Medicine, Hershey Medical Center, with Dr. Robert Bonneau studying the effects of stress on the immune response to Herpes Simplex Virus. Ms. Gussow began her graduate career at Arizona State University in Tempe, Arizona, with Dr. David Bloom before transferring to the University of Florida where she has completed her doctoral studies.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul J. Reier
Mark F. Overstreet Professor of Neuroscience

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard C. Condit
Professor of Molecular Genetics and Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 2004

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